The Journal of
CLINICAL
ENDOCRINOLOGY

VOLUME 10 JANUARY, 1950 NUMBER 1
Copyright 1950 by the Association for the Study of Internal Secretions

ON PIPERIDYLMETHYL-BENZODIOXANE (933-F), HYPERTENSION, AND PHEOCHROMOCYTOMA*

EVAN CALKINS, M.D., GEORGE W. DANA, M.D., JOHN C. SEED, M.D. AND JOHN EAGER HOWARD, M.D.

From the Department of Medicine, The Johns Hopkins University and Hospital, Baltimore, Maryland; and the Medical Division, Army Chemical Center

THERE has been much recent interest in the so-called "adrenolytic" drugs. It is now believed that these compounds inhibit the effects of epinephrine and allied pressor agents by occupying the epinephrine-specific receptor (1). The majority of the epinephrine inhibitors (*e.g.*, the benzodioxanes, ergotoxine, yohimbine, and the phenoxyethylamines) appear to combine reversibly with the receptor, thereby competing with epinephrine. As a possible basis for this competition, a common chemical configuration (C O C C N) has been observed in all the competitors mentioned, which may correspond to a similar configuration (H O C C N) in ephinephrine.[1] Competition implies that the degree of inhibition is proportional to the amount of inhibitor used and any degree of inhibition may be overcome by an excess of pressor agent.

Dibenamine, on the other hand, is thought to combine *irreversibly* with the epinephrine-specific receptor (2) and once inhibition by this compound has been established, it cannot be overcome by an excess of pressor agent. The combination of dibenamine with the receptor can, however, be prevented by previously filling the receptor. Thus, when benzodioxane, whose

Received for publication July 2, 1949.

* Read at the Annual Meeting of the Association for the Study of Internal Secretions, Atlantic City, N. J., June 3, 1949.

[1] Experiments in support of these theories were performed at the Army Chemical Center by one of us (J.C.S.) and will be reported in detail elsewhere.

action is short lived, is administered just before dibenamine, which is effective for several days, the organism becomes sensitive again to epinephrine within several hours (1).

Piperidylmethyl-benzodioxane (933-F) is the epinephrine competitor which has received the greatest clinical and pharmacologic attention to date. Although there has been considerable controversy concerning its properties, certain facts seem well substantiated. It is relatively nontoxic and is rapidly destroyed or excreted. Its chief side effect, central nervous system excitement, becomes pronounced in unanesthetized animals only after the administration of large doses (10 mg./kilo) (3). Such excitement is usually preceded by nausea and vomiting, symptoms which are useful warnings of more serious toxicity. The dosage suggested by Goldenberg, Snyder and Aranow (4), and used by us, is sufficiently small (0.25 mg./kilo) to provide a considerable margin of safety. 933-F is stable as a dry powder or when dissolved in physiologic saline, and it can be sterilized by autoclaving or boiling.

933-F, like other epinephrine competitors, will inhibit the pressor effect and most of the other effects of epinephrine and allied compounds. It will not, however, alter epinephrine's secondary vasodilating action, and this fact explains "epinephrine reversal," the actual lowering of blood pressure which occurs when a competitor is administered simultaneously with epinephrine (3, 5). Nor-epinephrine, which has recently been found to be present in large quantities in certain pheochromocytomas (6), has negligible vasodilating action. Hence, when 933-F is administered simultaneously with nor-epinephrine, the blood pressure may rise only slightly or not at all, but will not fall (7, 8).

Certain experiments suggest that 933-F, or the closely allied benzodioxane 883-F, will not only inhibit the pressor effects of epinephrine, but will also block the hypertension due to intermittent sympathetic stimulation or increased sympathetic tone (9, 10). In evaluating such experiments it must be borne in mind that unless the adrenal glands have been removed or excluded from the general circulation, stimulation intended to be confined to the sympathetic nervous system may also evoke a discharge of circulating epinephrine. From studies on preparations in which this precaution has been observed, it is clear that small doses of benzodioxane which will easily "reverse" the hypertensive effects of epinephrine, will exert little if any effect on the hypertension caused by direct sympathetic stimulation (7, 11). It has also been shown that 883-F, in doses as large as 5 mg./kilo, will not lessen the blood pressure of dogs with chronic renal hypertension resulting from the Goldblatt operation (10).

It seems altogether likely, therefore, on theoretical and experimental grounds, that the use of 933-F should identify hypertension due to pheo-

chromocytoma, as suggested by Goldenberg (4), if the hypertension resulting from these tumors is due to the direct effects of an excess circulating epinephrine, nor-epinephrine or allied compound. In view of the difficulties of diagnosing pheochromocytomas, particularly those associated with a sustained hypertension (12), the value of such a test is apparent.

This report deals with the administration of 933-F to 120 patients with hypertension. The bulk of the material was furnished by Dr. Fourneau, through the courtesy of Dr. D. O'Brien of the Rockefeller Institute in Paris. More recently 933-F has been provided by Merck and Company, in 20-mg. ampoules. Chemical identity of the former material was checked by Dr. Kenneth C. Blanchard, and the physiologic activity of both lots was confirmed by experiments on dogs and normal humans, who were rendered artificially hypertensive by intravenous infusions of commercial epinephrine. Adult patients were all given 20 mg. of the drug dissolved in 2 cc. of sterile saline, intravenously. The dosage in the children was calculated on the basis of 10 mg. per square meter of body surface. In all instances the drug was given over a two-minute period. Although a preliminary intravenous drip was used at first, it was soon found that this was not necessary in most adult patients. The patients were, however, rested until their blood pressure had become stabilized before the test was performed. The benzodioxane was then injected directly into the vein by means of a syringe, a two-minute interval being allowed to elapse between the venipuncture and the beginning of the injection.

Eight patients with apparent organic hypertension were studied. This group consisted of 2 patients with congenital polycystic kidneys, 2 with hypertension of pregnancy, 2 with chronic pyelonephritis, and 1 each with chronic glomerulonephritis and Cushing's disease. None of these responded to benzodioxane with a fall in blood pressure. In 3 the pressure rose immediately after administration of the drug, in the patient with Cushing's disease climbing from 160/100 to 235/130.

The remaining 112 hypertensive patients were referred for consultation because of suspicion of pheochromocytoma. Many of these had been observed by their physicians to have extremely labile blood pressures. Sixteen patients complained of sudden spontaneous attacks closely resembling those suffered by patients with pheochromocytoma (13). These patients, however, did not all have rises in blood pressure associated with their attacks, and in none was vomiting or blanching of the skin a feature.

In only 2 of the total group was administration of 933-F followed by a prompt fall in blood pressure of sufficient extent and duration to be judged as a *positive* result. These patients will be reported in detail in a subsequent section. In 7 others, who were particularly anxious about the test and whose blood pressure was temporarily elevated above their norm, injec-

tion of the drug was followed by a fall of over 20 mm. of Hg, systolic. In none of these was the diastolic fall more than 10 mm. of Hg, however, and all of them exhibited negligible responses when the test was repeated under more reassuring circumstances. In the remaining patients, the drug produced either a negligible blood pressure change or a rise. Although this rise was occasionally pronounced, as in the individual with Cushing's disease just mentioned, it was never as sudden or distressing as that caused by the injection of histamine in a patient with a pheochromocytoma (14, 15). Those patients who had the most labile resting blood pressure were not necessarily the ones who responded to 933-F with the greatest elevations of blood pressure.

In addition to the blood pressure responses noted, almost all the patients developed a tachycardia; and premature systoles, with or without compensatory pauses, were occasionally encountered. Virtually all patients became flushed, and a peculiar sighing respiration was frequently observed, similar to that seen in neurocirculatory asthenia. Several exhibited a fine tremor and in 1 patient fasciculations of the muscles of the shoulder and neck were observed.

Subjective response to the drug varied widely. Almost all of the patients complained of palpitation, nervousness and a sense of warmth. A few felt faint or giddy and complained of cold, tingling extremities. Occasionally a patient became panicky, burst into tears and cried out that he felt about to die. Those who reacted in this manner were not necessarily the ones who had appeared to be the most unstable emotionally.

Of the 16 patients with a history of repeated attacks mimicking those caused by pheochromocytomas, 9 experienced reduplication of the symptoms following administration of the 933-F. Many of these said "that medicine gave me just the sort of attack I came to get rid of." They did not necessarily have an associated rise in blood pressure following administration of the drug. Most of them were also given a histamine test (14), but this drug failed to produce reduplication of the spontaneous attacks in any.

A summary of the observations on the 2 patients who showed positive results with the benzodioxane tests follows:

Case 1

E. S., (J.H.H. #468773), a 37-year-old white laborer, was referred to Dr. H. M. Thomas, Jr. because of what was thought to be malignant hypertension.[2] Five years before admission he suddenly developed blurred vision, for which he was hospitalized elsewhere. Blood pressure was found to be 280 mm. of Hg and there was papilledema, hypertensive retinopathy, hyperglycemia and glycosuria. During a six months' rest, all of these abnormalities subsided, and he worked asymptomatically until six weeks before

[2] We are indebted to Dr. Thomas for permission to study this case, which is reported in detail elsewhere by Davis, Hull and Vardell (16).

admission. He then noted recurrence of blurred vision and experienced anorexia, weight loss, weakness, and frequent profuse sweats. Physical examination disclosed the following positive findings: The skin was peppered with lesions characteristic of neurofibromatosis. Resting blood pressure ranged between 150/110 and 185/135, the pulse between 70 and 120. There was marked retinopathy, with narrowed arterioles, moderate A-V nicking, a few scattered exudates, and central and macular degeneration. The heart was not enlarged, but the precordium was over-active, and there were systolic murmurs in both the mitral and aortic areas. There were no palpable abdominal masses, and pressure over the abdomen and costovertebral angles did not alter the blood pressure.

Blood morphology, urine and stool were normal. Serologic tests for syphilis were negative. Serum NPN, urea clearance and renal concentrating capacity were normal. The B.M.R. was plus 49 and plus 54 per cent, with a serum cholesterol of 335 mg. per 100 cc.

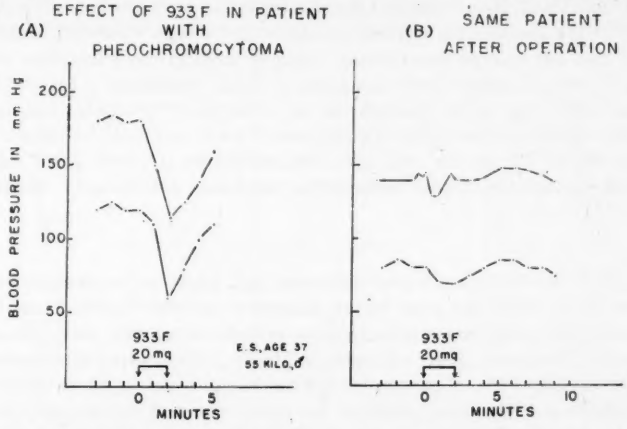

FIG. 1. (E. S.): This chart depicts the changes in systolic and diastolic blood pressure of a patient with subsequently proven pheochromocytoma, following the intravenous injection of 20 mg. of 933-F, (A) before, and (B) thirteen days after surgical removal of the tumor.

Fasting blood sugar was 140 mg. per cent and the blood glucose curve, following oral administration of 100 grams of glucose, disclosed the slow fall characteristic of diabetes mellitus. Deep sedation with sodium amytal caused the pressure to fall to 110/80. Intravenous injection of 0.025 mg. of histamine base resulted in a rise in blood pressure from 160/115 to 290/180 in sixty seconds, and the patient experienced a severe headache followed by a drenching sweat. The cold pressor test caused an elevation in blood pressure from 175/125 to 225/150. On two occasions the patient was given 20 mg. of 933-F intravenously. On both occasions the blood pressure fell approximately 80 mm. systolic and 60 mm. diastolic within three minutes, and did not return to its previous level for ten minutes. The results of one of these tests are shown in Figure 1A. During the test the patient's pulse rate rose from 88 to 120 and premature contractions and later bigeminal and trigeminal rhythm were evident. The patient complained of flush and displayed deep sighing respirations.

At operation, which was performed by Drs. J. A. C. Colston and W. W. Scott, an encapsulated tumor, the size of a golf ball, was found and removed from the left postadrenal area. Immediately after the pedicle was clamped, the blood pressure fell to imperceptible

levels. A dose of 1.5 mg. of epinephrine dissolved in 15 cc. of saline was injected intravenously during the course of approximately two minutes, but produced only a transitory elevation in blood pressure. A solution of epinephrine in glucose and saline, 1 mg. per 100 cc., was therefore administered by slow intravenous drip, the rate being adjusted so that the blood pressure remained approximately 100/60. During the twenty-eight hours in which this support was needed, a total of 50.4 mg. of epinephrine was administered. The blood pressure eventually stabilized at approximately 130/80. Fasting blood sugar, eight days after operation, was 84 mg. per cent, with a normal glucose tolerance curve. The B.M.R. was plus 17 per cent. Repetition of the histamine test produced a rise in blood pressure from 125/80 to 130/85. Following intravenous injection of 20 mg. of 933-F, the blood pressure fell only 10 mm. of Hg, systolic and diastolic, and returned to normal in two minutes (Fig. 1, B). Pathologic examination of the tumor confirmed the diagnosis of pheochromocytoma. Biopsy showed the skin lesions to be typical neurofibromata.

Extracts of the tumor with physiologic saline and Ringer's solution were tested for epinephrine and nor-epinephrine content, using isolated rabbit's intestine and isolated rat uterus. It was estimated that each gram of tumor contained a total of 2.5 mg. of epinephrine and 12 mg. of nor-epinephrine. In a dog under nembutal anesthesia it was demonstrated that the pressor effect of an extract of the tumor could be almost completely inhibited by 933-F. The pressor responses obtained before and after 933-F corresponded to the ratio of epinephrine and nor-epinephrine previously determined in the gland.

Case 2

S. R., (J.H.H. #A-64094), a 7-year-old school girl, began to lose weight and to sweat profusely in June, 1947, one year before admission to the Harriet Lane Home. Her mother observed a rapid pulse. Although these symptoms subsided after several months, they recurred in December, 1947, and again in March, 1948. During these latter episodes, the patient complained also of pain in her left flank, and a mass became visible in the left side of the abdomen. The blood pressure was found elevated, but no paroxysms of palpitation, blanching or sweating occurred. Physical examination revealed a markedly emaciated, pale, listless girl with cold moist skin and a generalized sudaminal rash. The pupils were unduly dilated but reacted normally. Fundi showed narrowed arterioles, A-V nicking, and several small hemorrhages and exudates. The thyroid was not enlarged. The heart was enlarged, the precordium very active, and a loud pulmonic systolic murmur was present. Blood pressure ranged between 190/140 and 220/190, and the pulse between 80 and 140. There was a hard, non-tender, immovable mass, the size of a grapefruit, in the left upper quadrant, manipulation of which failed to elicit blood pressure changes. The edge of the liver and spleen could also be felt.

Laboratory findings: The hemoglobin was 15.5 Gm. and the white cell count 11,200 with 58 per cent polymorphonuclear leucocytes, 37 per cent lymphocytes, 2 per cent monocytes, 2 per cent eosinophiles and 1 per cent basophiles. Urinalysis showed nothing abnormal except for albuminuria. Urine concentration and the results of a PSP test were normal. Blood NPN and fasting sugar were normal in amount and the glucose tolerance curve was normal. The B.M.R. was plus 4 per cent. An electrocardiogram showed left axis deviation. A roentgenogram showed an enlarged heart. Intravenous and retrograde pyelograms revealed downward displacement of the left kidney. The histamine test was not performed because of the high baseline blood pressure. The patient was given several doses of 933-F intravenously, ranging from 3 to 13 mg. each, during the course of two minutes. The fall in blood pressure was roughly proportional to the amount of drug used. Figure 2A depicts the response to 10 mg. of 933-F, a dose which, when calculated on the

basis of surface area, is the equivalent of 20 mg. of the drug in an adult. Coincident with this response was an increase in pulse rate from 130 to 250, a marked flush and nausea. After administration of the larger dose, 13 mg., the patient vomited.

At operation, which was performed by Drs. M. M. Ravitch and R. P. Higgins, Jr. on June 17, 1948, a large, irregular, retroperitoneal mass, apparently incorporating the left adrenal gland, was removed, together with the adherent left kidney and spleen. Fragments of the tumor which surrounded the inferior mesenteric vessels and invaded the posterior abdominal wall were left in situ. Minimal blood pressure changes were observed at operation, and supplementary epinephrine was not required. Immediately after the operation the blood pressure was 160/130, and it fell gradually until at discharge

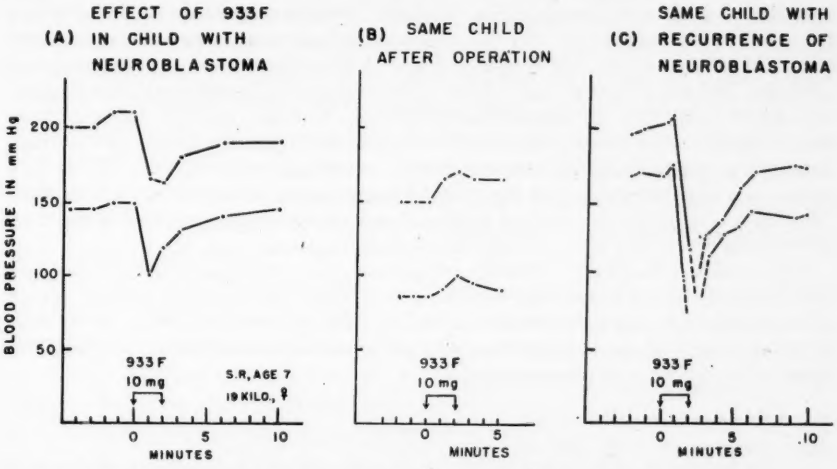

FIG. 2. (S. R.): This chart shows the changes in systolic and diastolic blood pressure of a 7-year-old child with neuroblastoma, following the intravenous injection of 10 mg. of 933-F, (A) before surgical removal of the tumor, (B) two and one half months after operation, and (C) upon development of metastases and recurrence of symptoms six months after operation.

three weeks later it did not exceed 140/95. Intravenous administration of 10 mg. of 933-F at this time produced negligible change in blood pressure.

The microscopic appearance of the tumor was described by Dr. A. R. Rich as follows: "This tumor is a typical neuroblastoma. In some areas, however, the cells are larger than in ordinary neuroblastoma and in some midway between ganglion cells and neuroblasts." The final diagnosis was large-cell neuroblastoma. Chromate stain gave negative results. Extracts of several portions of the tumor were tested for epinephrine and nor-epinephrine by the methods mentioned above and by determining the pressor effect caused by intravenous administration of these extracts to anesthetized dogs. The total amount of epinephrine-like substance was approximately one gamma per gram of tumor.

Following discharge, the patient had no more pains or sweating and began to gain weight. A total of 3,200 roentgen units was administered over the tumor area during a three-week period. When the patient was seen in September, two and a half months after discharge, the blood pressure was found to range between 110/80 and 150/90. There was

marked improvement in the appearance of both fundi. The heart was normal in size and no murmurs were heard. No abdominal mass could be felt. The liver edge was barely palpable. Hematologic and urinary findings, and blood nonprotein nitrogen were in the normal range. Roentgenograms of the skull and long bones showed nothing abnormal. Intravenous injection of 0.006 mg. of histamine base caused a change in blood pressure from 150/90 to 160/85. Repetition of the benzodioxane test produced an elevation of the blood pressure from 150/85 to 170/100 (Fig. 2 B).

The patient continued to feel well for another month. Then intermittent sweats and weight loss returned, and she complained of pain in her right costovertebral angle and shoulder. Upon readmission on December 17, 1948, she appeared pale, anxious and emaciated. There was generalized lymphadenopathy and the liver edge extended three fingerbreadths below the right costal margin. The blood pressure ranged between 190/140 and 220/190. The extremities were very pale and cold, and pulsation in the dorsalis pedis vessels could barely be felt. The hemoglobin was 19 Gm. per 100 cc., the red cell count 6,000,000, and the white cell count 9,000. Urine was normal except for a trace of albumen. Blood nonprotein nitrogen and serum protein, albumen, globulin, calcium, phosphorus, alkaline phosphatase, cephalin flocculation and thymol turbidity were normal. Roentgenograms of the skeletal system showed no metastases. As shown in Figure 2 C, intravenous administration of 10 mg. of 933-F caused a fall in blood pressure from 200/180 to unobtainable levels, returning to normal only after several hours. During this time the sweating ceased and the previously blanched skin appeared rosy.

Unfortunately, the patient suffered a cerebrovascular accident several days later, and died. Autopsy showed widespread metastases involving abdominal and thoracic lymph nodes, pleura, liver, and right periadrenal tissues. The microscopic appearance was similar to that described previously. Extracts of the metastases revealed that the tissue contained no epinephrine or nor-epinephrine.

DISCUSSION

In the first case (E.S.) presented here, the association of the tumor with the patient's hypertension and other symptoms is quite evident. Pathologically the tumor was a typical pheochromocytoma, and pharmacologic studies indicated that it contained a large amount of pressor substance, chiefly nor-epinephrine. It seems very plausible that in cases of this sort —in which the blood pressure is reduced to normal or subnormal by 933-F, is further reduced to unobtainable levels when the pedicle is clamped, and later is stabilized at a normal level—the hypertension is due to the *direct* effects of the pressor substance on the arteriolar bed and the myocardium.

In the second case (S.R.), however, the blood pressure responses are most puzzling. The fact that the hypertension and associated symptoms almost totally disappeared following removal of most of the tumor, only to recur with the development of remote metastases, strongly suggests that the tumor stood in relation to the child's symptoms as cause and effect. Nevertheless, extracts of the tumor contained insignificant amounts of pressor substance, and on pathologic examination the tumor was found to be a large cell neuroblastoma, rather than a pheochromocytoma.

It is possible, to be sure, that although the tumor appeared to contain no

pressor material, it may have made and excreted it. In this connection it is interesting to note, as Dr. A. R. Rich pointed out, that this is not the first neuroblastoma seen at the Johns Hopkins Hospital which was associated with hypertension. In 1933, an eight-year-old colored boy (C.W., J.H.H. #43415) was admitted because of a cerebrovascular accident. He was found to have a blood pressure of 170/130, an enlarged heart, albuminuria, and azotemia, and he died shortly after admission. Partial autopsy revealed a retroperitoneal and retropleural neuroblastoma, which also involved the mediastinal, aortic and splanchnic lymph nodes and the right femur. Although the records of 12 other children with proven neuroblastoma were also studied, none of them was found to have had hypertension or any stigmata of hypertension; and none of the tumors, upon microscopic examination, appeared to contain epinephrine-secreting tissue. That a neuroblastoma can contain pheochromocytomatous elements, however, is substantiated by one report in the literature (17), and it is possible that S. R.'s tumor contained small islands of adult chromaffin tissue which did not happen to be located in any of the many areas studied, microscopically or pharmacologically.

In any case, on both occasions when the patient S. R. was hypertensive the results with the benzodioxane test appeared clearly positive; it thus accurately foretold that following removal of the tumor, the blood pressure would fall, at least to some extent. Nevertheless, there were significant differences between the blood pressure responses of this patient and those of the first patient (E.S) previously presented. The test dose of benzodioxane preoperatively lowered the blood pressure from 210/150 to 160/110 but did not reduce it to normal; the pressure remained relatively stable during operation, even when the pedicle of the tumor was clamped; and the pressure did not fall to normal until over three weeks after the operation.

Other patients, with proven pheochromocytomas, have followed the same pattern. In Goldenberg, Snyder, and Aranow's patient #2, the test dose of benzodioxane lowered the blood pressure only to 154/80, and two weeks after removal of the tumor the pressure was still considerably elevated at 160/116 (4). Similarly Wilkins reported a patient with subsequently proven pheochromocytoma in whom 933-F produced a negligible fall in pressure and whose pressure remained relatively steady throughout the operation, only falling to normal five or ten days postoperatively (18).

It is possible that in cases of this sort, pheochromocytomas may cause hypertension through some mechanism other than the direct action of their secretions on the arteriolar bed or myocardium. One is reminded of the gradual fall in blood pressure following the removal of the tumor from individuals with Cushing's syndrome, at least after the initial acute adrenal insufficiency has passed. Epinephrine is thought to cause the pituitary to

discharge ACTH (19), and thus chronic epinephremia might induce chronic hypercorticism. The young patient of Neff and associates (20), who had many of the features of Cushing's disease and yet had a tumor which was found microscopically to be a pheochromocytoma, has always puzzled us, but might be thus explained.[3]

SUMMARY AND CONCLUSIONS

1. 933-F competes with epinephrine and allied compounds for an epinephrine-specific receptor, and, in 20-milligram doses, will disclose that component of hypertension which is directly due to such compounds.

2. The benzodioxane test was performed on 120 hypertensive patients, of which all but 2 gave a negative response. The type and severity of the side effects of 933-F in this group could not be correlated with blood pressure response or with the individual's emotional pattern.

3. One patient who gave a clearly positive response to 933-F was subsequently proven to have a pheochromocytoma, by surgical removal of this tumor with complete relief of his symptoms. Extracts of the tumor contained large amounts of nor-epinephrine, and the pressor effect of these extracts in dogs was inhibited by 933-F.

4. A second patient who gave a positive response to 933-F was found to have a large-cell neuroblastoma. Nevertheless, her hypertension was relieved following removal of the tumor, only to recur with the development of metastases.

5. In certain patients with proven pheochromocytomas, the results of the benzodioxane test may be equivocal or negative, the blood pressure may remain stable throughout operation, and return to normal only gradually thereafter. In these patients, it is postulated that the pheochromocytomas may produce hypertension indirectly by stimulating the pituitary and adrenal, rather than directly by the effect of their secretions on the arteriolar bed or cardiac musculature.

REFERENCES

1. SEED, J. C., and MCKAY, E. A.: Inhibition by piperidinomethyl-3-benzodioxane (933-F) of epinephrine vasopressor blockade produced by dibenzyl-β-chlorethylamine, *Proc. Soc. Exper. Biol. & Med.* **70**: 724, 1949.
2. NICKERSON, M., and GOODMAN, L. S.: Adrenergic blockade, with special reference to dibenamine, *Fed. Proc.* **7**: 397, 1948.
3. BOVET, D., and SIMON, A.: Recherches sur l'activité sympatholytique des dérivés

[3] In the discussion which followed the reading of this paper at the annual meeting of the Society for the Study of Internal Secretions, Dr. Randall Sprague reported that he had studied the urinary 11-oxysteroids and 17-ketosteroids in a patient with a pheochromocytoma and found both to be elevated.

de l'aminométhylbenzodioxan, *Arch. internat. de pharmacodyn, et de thérap.* **55:** 15, 1937.

4. Goldenberg, M.; Snyder, C. H., and Aranow, H., Jr.: New test for hypertension due to circulating epinephrine, *J.A.M.A.* **135:** 971, 1947.
5. Dale, H. H.: On some physiological actions of ergot, *J. Physiol.* **34:** 163, 1906.
6. Goldenberg, M.; Faber, M.; Alston, E. J., and Chargaff, E. C.: Evidence for the occurrence of nor-epinephrine in the adrenal medulla, *Science* **109:** 534, 1949.
7. Melville, K. I.: Antisympathomimetic action of dioxane compounds (F-883 and F-933,) with special reference to vascular responses to dihydroxyphenyl ethanolamine (arterenol) and nerve stimulation, *J. Pharmacol. & Exper. Therap.* **59:** 317, 1937.
8. Zunz, E., and Bonnyns, R.: Recherches sur les effets du diéthylaminométhyl-3-benzodioxane, du pipéridométhyl-3-benzodioxane, et du paraméthoxydiéthylaminoéthyl phénol sur l'hypertension et l'aprée provoquées par les sympathomimétique chez le chien, *Arch. internat. de pharmacodyn, et de thérap.* **58:** 108, 1938.
9. Fourneau, E., and Bovet, D.: Recherches sur l'action sympathicolytique d'un nouveau dérivé du dioxane, *Arch. internat. de pharmacodyn, et de thérap.* **46:** 178, 1933.
10. Bing, R. J., and Thomas, C. B.: Effect of dioxane derivatives, 883 and 933-F on normal dogs and on animals with neurogenic and renal hypertension, *J. Pharmacol. & Exper. Therap.* **83:** 21, 1945.
11. de Vleeschhouwer, G.: Au sujet de l'action du diéthylaminométhyl-3-benzodioxane (F. 883) et du pipéridométhyl-3-benzodioxane (F. 933) sur le système circulatoire, *Arch. internat. de pharmacodyn, et de thérap.* **50:** 251, 1935.
12. Green, D. M.: Pheochromocytoma and chronic hypertension, *J.A.M.A.* **131:** 1260, 1946.
13. Howard, J. E., and Barker, W. H.: Paroxysmal hypertension and other clinical manifestations associated with benign chromaffin tumors (phaeochromocytomata), *Bull. Johns Hopkins Hosp.* **61:** 371, 1937.
14. Roth, G. M., and Kvale, W. F.: Tentative test for pheochromocytoma, *Am. J. M. Sc.* **210:** 653, 1945.
15. Calkins, E., and Howard, J. E.: Bilateral familial phaeochromocytomata with paroxysmal hypertension: successful surgical removal of tumors in two cases with discussion of certain diagnostic procedures and physiological considerations, *J. Clin. Endocrinol.* **7:** 475, 1947.
16. Davis, F. W., Jr.; Hull, J. G., and Vardell, J. C.: Pheochromocytoma with neurofibromatosis, *Am. J. Med.* (in press).
17. Wahl, H. R., and Robinson, D. W.: Neuroblastoma of mediastinum with pheochromoblastomatous elements, *Arch. Path.* **35:** 571, 1943.
18. Wilkins, R. W.: Personal communication to one of the authors.
19. Long, C. N. H.: Conditions associated with secretion of the adrenal cortex, *Fed. Proc.* **6:** 461, 1947.
20. Neff, F. C.; Tice, G. M.; Walker, G. A., and Ockerblad, N.: Adrenal tumor in female infant with hypertrichosis, hypertension, overdevelopment of external genitalia, obesity, but absence of breast enlargement, *J. Clin. Endocrinol.* **2:** 125, 1942.

PRODUCTION OF ENDOGENOUS "SALT-ACTIVE" CORTICOIDS AS REFLECTED IN THE CONCENTRATIONS OF SODIUM AND CHLORIDE OF THERMAL SWEAT*

JEROME W. CONN, M.D. AND LAWRENCE H. LOUIS, SC.D., WITH THE TECHNICAL ASSISTANCE OF SHIRLEY WARK, M.S. AND BETTY SPRUNGER

From the Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan

THE evidence given in this article appears to be adequate to establish the facts i) that the concentrations of sodium and chloride in sweat are governed by activity of the adrenal cortex, ii) that these concentrations reflect the degree of activity of the "salt-active" corticosteroids (desoxycorticosterone-like[1] corticoids), iii) that administration of pituitary adrenocorticotropic hormone (ACTH) to normal people is capable of stimulating a simultaneous increase in the production of all three types of physiologically active adrenal corticosteroids, *i.e.*, the DCA-like corticosteroids, the 11 and 11–17 oxysteroids and those excreted as 17-ketosteroids, and iv) that the complicated process of metabolic acclimatization to heat is accomplished by increased secretory activity of the adrenal cortices which have in turn been activated by pituitary adrenocorticotropin. Preliminary observations indicate the probability that the sweat electrolyte pattern can be used in clinical and in experimental medicine as an indication of either diminished or of excessive adrenal production of "salt-active" corticosteroids.

It has been reported from this laboratory (1) (2) that there exists in man an adaptive mechanism for the conservation of body salt whereby the sweat glands, under the stress of impending depletion of body salt, are capable of producing sweat which is extremely dilute in sodium and chloride. This mechanism is activated in normal men during the process of acclimatization to heat. It is activated further and with even greater intensity in fully acclimatized men working in heat, when their salt intakes are abruptly reduced.

It was observed subsequently (3) that each time that this mechanism for reducing sweat salt concentration is activated acutely, a period of negative

Received for publication June 9, 1949.

* This work represents part of a project financed by the Research Grants and Fellowships Division of the U. S. Public Health Service.

[1] Hereafter referred to as DCA-like corticoids.

nitrogen balance occurs. This is always the case during the process of acclimatization to heat. It is true also in fully acclimatized men who have long since attained nitrogen equilibrium, but in whom the salt intake is abruptly reduced. The constant association of negative nitrogen balance with a physiologic attempt to conserve body salt suggested i) that adrenal cortical activity was involved in the mechanism, ii) that under conditions of our experiments, the stimulus to conserve body salt constituted the primary activator of the adrenal mechanism (addition of sodium chloride to the diet inhibited the mechanism), and iii) that the decrease in salt concentration of the sweat represented an effect of adrenal cortical steroids upon the function of the sweat glands.

It was found next (3) that administration of desoxycorticosterone acetate (DCA) to normal men living in a temperate climate resulted in a sharp fall in the concentration of sodium and chloride of sweat. This, as expected, was not accompanied by negative nitrogen balance. Similar experiments in acclimatizing or in fully acclimatized men indicated that the mechanisms through which man adapts to heat can be interfered with by administration of DCA. The results were highly suggestive of a depressing influence of DCA upon the pituitary-adrenal mechanism.

The present study, involving the administration of purified ACTH,[2] was designed primarily to establish the validity of the thesis that metabolic acclimatization to heat is acquired via pituitary-adrenal hyperactivity. In the course of these experiments it became clear that the electrolyte composition of thermal sweat was reflecting changes in adrenal cortical function (4).

METHODS

Two normal young women (A. M. and M. W.) and 2 normal young men (R. S. and D. E.) volunteered as subjects for the long-term balance studies. All were fed a constant, carefully weighed diet for thirty-two consecutive days. The diet for A. M., M. W. and D. E. contained 99 Gm. of protein, 300 Gm. of carbohydrate and 176 Gm. of fat. Analysis showed it to contain 15.75 Gm. of nitrogen, 4.4 Gm. of sodium and 4.8 Gm. of potassium. The diet of R. S. differed from that of the other subjects only in that it contained 197 Gm. of fat, 4.63 Gm. of sodium and 4.86 Gm. of potassium.

Following a suitable baseline period, each subject was given adrenocorticotropic hormone (ACTH) intramuscularly every eight hours for from eight to ten days (*see* dosage levels in Figures). Recovery from the metabolic effects of ACTH was followed for from seven to thirteen days after cessation of administration of ACTH.

[2] We are indebted to Dr. J. R. Mote, The Armour Laboratories, Chicago, Ill., for the purified adrenocorticotropic hormone used in these studies.

Chemical methods. Determinations performed, which are connected with the report, were urinary and sweat sodium (5) and chloride (6), urinary potassium (7), uric acid (8) (9), glucose (10), 17-ketosteroids (11), and nitrogen by the macro-kjeldahl method.

Collection of samples of sweat. In the present studies samples of sweat were collected during a two-hour resting exposure of the entire body to humid heat (95 F. and 90 per cent rel. humidity). Sweat samples were collected simultaneously from both the abdomen and the hand-plus-forearm during each exposure to heat. The technic of collection of such samples has been described elsewhere (12).

RESULTS AND COMMENTS

The results are shown graphically in Figures 1 through 8.

Effect of ACTH upon sodium and chloride concentrations of thermal sweat

It is clear that administration of ACTH produces in the sweat of normal people a progressively decreasing concentration of sodium and chloride and that the lowest concentrations of these elements are observed on the last day of ACTH administration or on the day following the last injection of the hormone (Figs. 1, 3, 5 and 7). In the recovery period the pattern is that of a rebound in these concentrations to or above the baseline values, followed by temporary secondary depression. These results are precise reproductions of those which we have observed to occur upon administration to normal individuals of 7 to 20 milligrams per day of desoxycorticosterone acetate (2) (3).

Although the effect of ACTH and of DCA upon electrolyte concentration of sweat is the same, the expected difference in the urinary excretion of 17-ketosteroids was observed. Whereas no significant increase of these compounds occurs during administration of DCA, a three to five-fold increase is observed when ACTH is employed. Thus, one observes (Figs. 1, 3, 5, and 7) two effects of excessive stimulation of the adrenal cortices with ACTH, namely, evidence of increased production of both androgenic corticosteroids and of DCA-like corticosteroids.

Effect of ACTH upon urinary excretion of sodium, chloride and potassium

In Figures 2, 4, 6 and 8 one recognizes the characteristic effects of desoxycorticosterone upon urinary excretion of electrolytes. It is to be emphasized, however, that these effects were produced by administration of ACTH. Figure 6 shows the most dramatic effect, subject R. S. having proved to react to ACTH with intense metabolic responses. On the first day of ACTH, potassium diuresis occurred. Urinary potassium then leveled out at approximate baseline values for the remainder of the ACTH period.

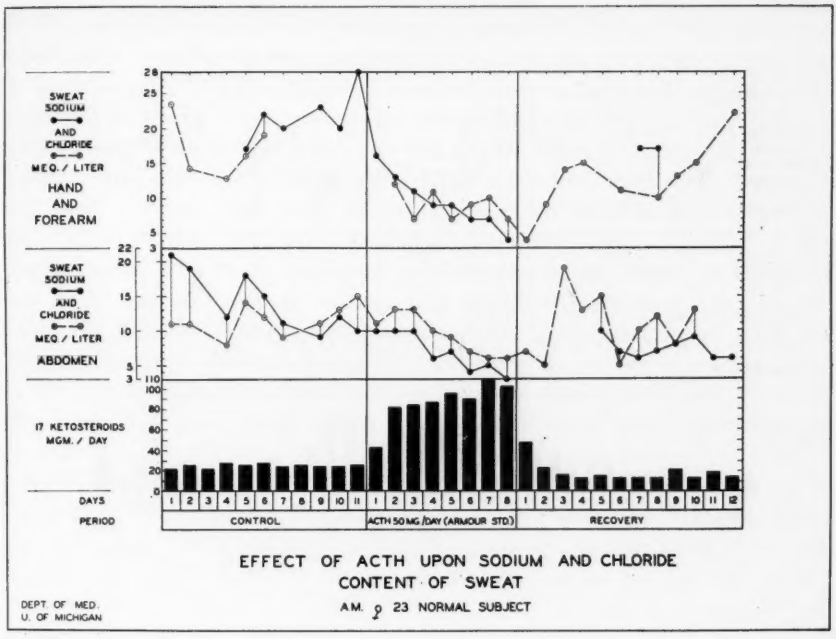
SWEAT SODIUM AND CHLORIDE MEQ. / LITER
HAND AND FOREARM
SWEAT SODIUM AND CHLORIDE MEQ. / LITER
ABDOMEN
17 KETOSTEROIDS MGM. / DAY
DAYS
PERIOD
CONTROL
ACTH 50 MG./DAY (ARMOUR STD.)
RECOVERY
EFFECT OF ACTH UPON SODIUM AND CHLORIDE CONTENT OF SWEAT
A.M. ♀ 23 NORMAL SUBJECT
DEPT. OF MED. U. OF MICHIGAN

FIGURE 1

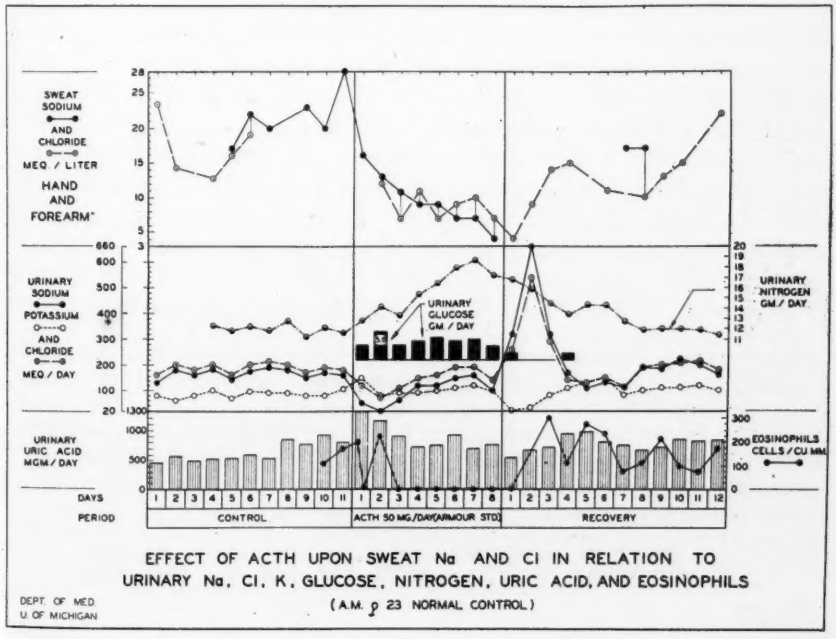
SWEAT SODIUM AND CHLORIDE MEQ. / LITER
HAND AND FOREARM
URINARY SODIUM POTASSIUM AND CHLORIDE MEQ. / DAY
URINARY GLUCOSE GM. / DAY
URINARY NITROGEN GM. / DAY
URINARY URIC ACID MGM. / DAY
EOSINOPHILS CELLS / CU MM.
DAYS
PERIOD
CONTROL
ACTH 50 MG./DAY (ARMOUR STD.)
RECOVERY
EFFECT OF ACTH UPON SWEAT Na AND Cl IN RELATION TO URINARY Na, Cl, K, GLUCOSE, NITROGEN, URIC ACID, AND EOSINOPHILS
(A.M. ♀ 23 NORMAL CONTROL)
DEPT. OF MED. U. OF MICHIGAN

FIGURE 2

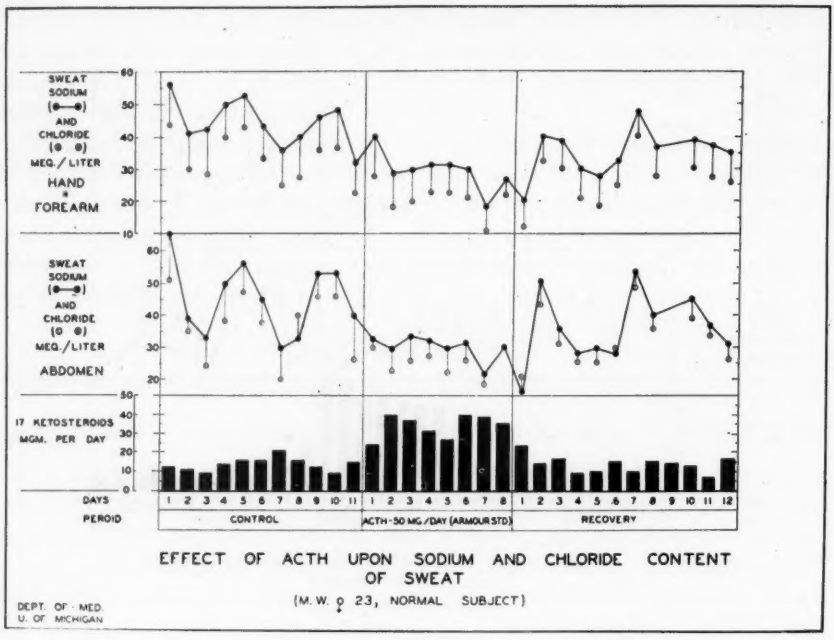
SWEAT SODIUM (●—●) AND CHLORIDE (○ ○) MEQ./LITER
HAND + FOREARM
SWEAT SODIUM (●—●) AND CHLORIDE (○ ○) MEQ./LITER
ABDOMEN
17 KETOSTEROIDS MGM. PER DAY
DAYS
PERIOD
CONTROL
ACTH-50 MG./DAY (ARMOUR STD.)
RECOVERY
EFFECT OF ACTH UPON SODIUM AND CHLORIDE CONTENT OF SWEAT
(M. W. ♀ 23, NORMAL SUBJECT)
DEPT. OF MED. U. OF MICHIGAN

FIGURE 3

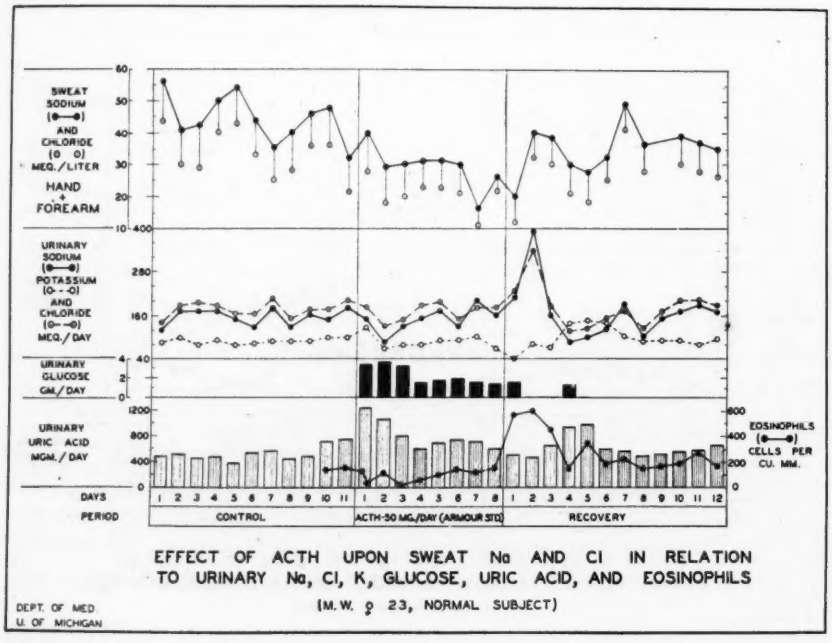
SWEAT SODIUM (●—●) AND CHLORIDE (○ ○) MEQ./LITER
HAND + FOREARM
URINARY SODIUM (●—●) POTASSIUM (○ - ○) AND CHLORIDE (○—○) MEQ./DAY
URINARY GLUCOSE GM./DAY
URINARY URIC ACID MGM./DAY
EOSINOPHILS (●—●) CELLS PER CU. MM.
DAYS
PERIOD
CONTROL
ACTH-50 MG./DAY (ARMOUR STD.)
RECOVERY
EFFECT OF ACTH UPON SWEAT Na AND Cl IN RELATION TO URINARY Na, Cl, K, GLUCOSE, URIC ACID, AND EOSINOPHILS
(M. W. ♀ 23, NORMAL SUBJECT)
DEPT. OF MED. U. OF MICHIGAN

FIGURE 4

Intense sodium and chloride retention occurred at once and continued for eight days with the development of obvious edema and a sharp increase in body weight. It is significant that much more sodium than chloride was retained, since this phenomenon is typical of the effect of desoxycorticosterone. On the ninth and tenth days of ACTH administration the characteristic "rebound despite continued administration" occurred. This was the most delayed "rebound" of urinary sodium and chloride observed, although it is clear that the rebound phenomenon began to occur on the fourth day of ACTH. In the other subjects "rebound" began on the third day of ACTH and urinary sodium and chloride levels were back to the baseline by the fifth day (Figs. 2, 4 and 8). Note, that in all cases no rebound occurs in the concentration of sodium and chloride of sweat so long as ACTH is administered. It becomes clear, then, that characteristic effects of DCA activity upon renal excretion of electrolytes are produced by ACTH, in that these effects are relatively transient and by the fifth to seventh days they no longer give any indication of excessive activity of DCA-like corticosteroids. On the other hand, the concentrations of sodium and chloride of sweat remain depressed for as long as either DCA or ACTH is administered.[3] Thus, it would appear that changes in the concentrations of these electrolytes in sweat reflect changes in the degree of activity of endogenous "salt-active" (DCA-like) corticosteroids.

To continue the analysis of the data on subject R. S. (Fig. 6), it is noteworthy that upon cessation of ACTH administration there occurred tremendous diuresis of sodium and chloride, and also great retention of potassium. The loss of sodium in the diuretic period exceeded considerably that for chloride. During this period of sodium diuresis and potassium retention body weight fell sharply, systolic blood pressure dropped below 100 mm. of mercury and postural hypotension was observed. In addition, marked lassitude, muscular weakness and mild anorexia developed (the diet was eaten completely, nevertheless). On the fifth recovery day the fasting blood sugar was 66 mg. per cent as compared with the average baseline value of 84 mg. per cent. All of these findings are highly suggestive of a short period of adrenal cortical insufficiency.

[3] Forsham *et al.* (13) have shown recently that Compound E decreases the sodium and chloride retaining effect of desoxycorticosterone in Addison's disease. It is likely that the "rebound" observed in normal people receiving either DCA or ACTH is produced by the endogenous production of Compound E or of a related steroid which acts competitively with desoxycorticosterone upon tubular reabsorption of sodium and chloride. The effect upon sweat electrolytes of Compound E, either alone or with desoxycorticosterone, has not been tested. The fact that DCA in normal subjects and in cases of Addison's disease, and ACTH in normal subjects, produce a sustained fall in the concentrations of sodium and chloride of sweat suggests that inhibition by other steroids of the desoxycorticosterone effect on the sweat glands does not occur.

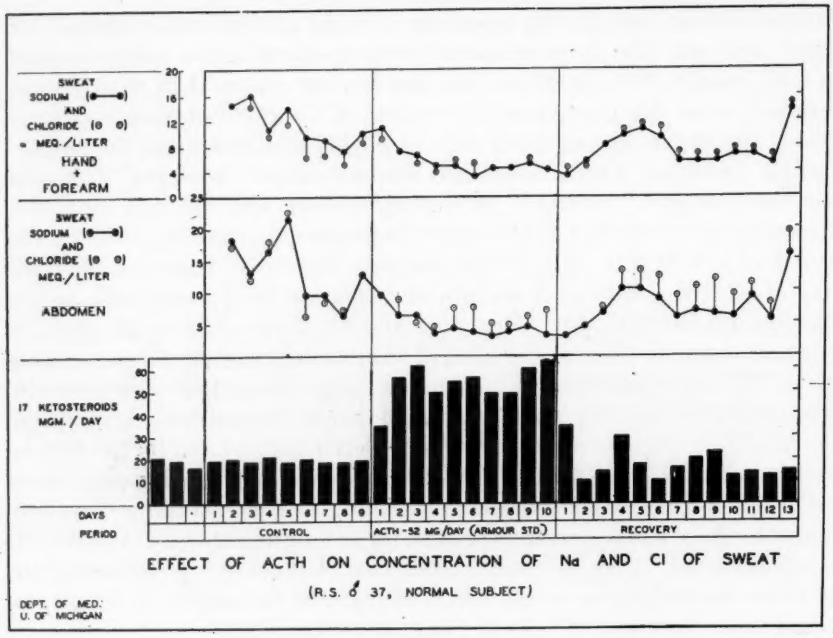
SWEAT SODIUM (●—●) AND CHLORIDE (○ ○) MEQ./LITER
HAND + FOREARM
SWEAT SODIUM (●—●) AND CHLORIDE (○ ○) MEQ./LITER
ABDOMEN
17 KETOSTEROIDS MGM. / DAY
DAYS
PERIOD
CONTROL
ACTH - 52 MG./DAY (ARMOUR STD.)
RECOVERY
EFFECT OF ACTH ON CONCENTRATION OF Na AND Cl OF SWEAT
(R.S. ♂ 37, NORMAL SUBJECT)
DEPT. OF MED.
U. OF MICHIGAN

FIGURE 5

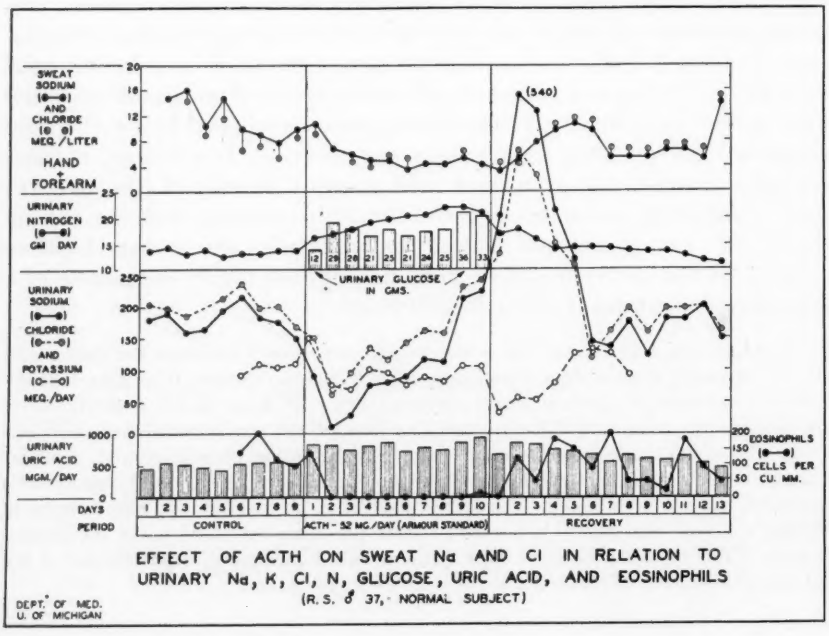
SWEAT SODIUM (●—●) AND CHLORIDE (○ ○) MEQ./LITER
HAND + FOREARM
(540)
URINARY NITROGEN (●—●) GM DAY
URINARY GLUCOSE IN GMS.
URINARY SODIUM (●—●) CHLORIDE (○---○) AND POTASSIUM (○—○) MEQ./DAY
URINARY URIC ACID MGM./DAY
EOSINOPHILS (●—●) CELLS PER CU. MM.
DAYS
PERIOD
CONTROL
ACTH - 52 MG./DAY (ARMOUR STANDARD)
RECOVERY
EFFECT OF ACTH ON SWEAT Na AND Cl IN RELATION TO URINARY Na, K, Cl, N, GLUCOSE, URIC ACID, AND EOSINOPHILS
(R.S. ♂ 37, NORMAL SUBJECT)
DEPT. OF MED.
U. OF MICHIGAN

FIGURE 6

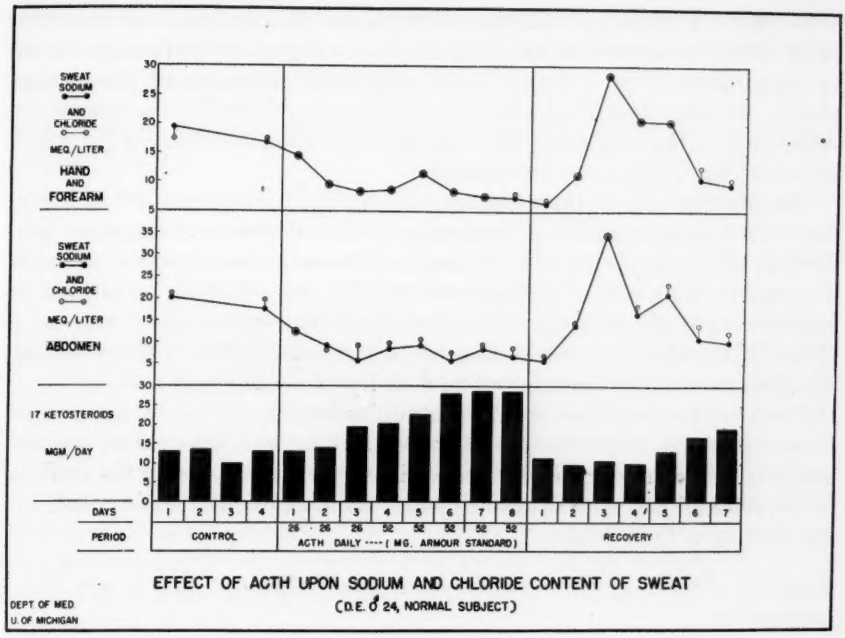

FIGURE 7

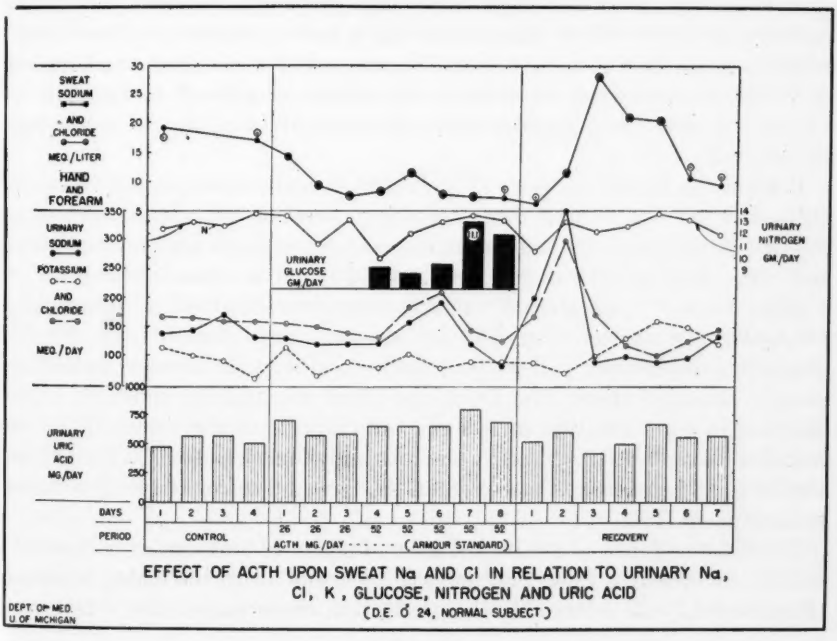
SWEAT SODIUM AND CHLORIDE MEQ./LITER
HAND AND FOREARM
URINARY SODIUM POTASSIUM AND CHLORIDE MEQ./DAY
URINARY GLUCOSE GM./DAY
URINARY NITROGEN GM./DAY
URINARY URIC ACID MG./DAY
DAYS
PERIOD
CONTROL
ACTH MG./DAY ------ (ARMOUR STANDARD)
RECOVERY
DEPT. OF MED. U. OF MICHIGAN
EFFECT OF ACTH UPON SWEAT Na AND Cl IN RELATION TO URINARY Na, Cl, K, GLUCOSE, NITROGEN AND URIC ACID
(D.E. ♂ 24, NORMAL SUBJECT)

FIGURE 8

On days six and seven of the recovery period one observes a spontaneous renal retention of sodium and chloride and a diuresis of potassium. In all of the subjects (Figs. 2, 4 and 8) this remarkable phenomenon occurred at about the same time and was followed one day later by the secondary depression of the concentration of sodium and chloride of sweat (to which reference has been made previously).

We interpret all of these changes during the recovery period as meaning that i) administration of exogenous ACTH depresses endogenous production of this hormone, ii) withdrawal of the exogenous hormone unmasks decreased production of endogenous ACTH, the metabolic consequence manifesting itself as temporary adrenal cortical insufficiency, and iii) a sharp rebound in the production of endogenous ACTH with secondary manifestations of increased cortical activity of a temporary nature.

From the point of view of this communication, the fact to be emphasized is that changes in the concentration of sodium and chloride of thermal sweat (collected under standard conditions) parallel changes in the activity of the adrenal cortex with respect to its elaboration of steroids which act like desoxycorticosterone.

Effect of ACTH upon metabolic processes influenced by C_{11} and $C_{11\text{-}17}$ oxysteroids

In Figures 2, 4, 6 and 8 are recorded data obtained upon urinary nitrogen, glucose and uric acid, and upon circulating polymorphonuclear cells of the eosinophilic type. These phenomena have been discussed in detail elsewhere (14, 15, 16). Some of these data are included here because they are of value in correlating temporally, the effects of adrenal elaboration of "11-oxysteroids" with those which indicate activity of the DCA-like corticosteroids.

Both of the female subjects (Figs. 2 and 4) had a spontaneous return of glycosuria on the fourth day, following cessation of administration of ACTH. Both also had a sudden increase of urinary uric acid on the fourth and fifth post-ACTH days. Simultaneously with these indications of a rebound in "11-oxysteroid" activity came evidence of a rebound in DCA-like steroid activity. Urinary excretion of sodium and chloride dropped considerably below the baseline values and urinary potassium greatly exceeded them. The DCA-like effect was quickly reflected in the decrease in concentration of sodium and chloride of the sweat. Thus the metabolic responses observed on the rebound (fourth post-ACTH day) are similar in all respects to those seen on the first and second days of administration of ACTH.

No clear evidence of a rebound in production of androgenic corticosteroids is observable in these 2 subjects (Figs. 1 and 3). In the males, however, (Figs. 5 and 7) the rebound in the production of androgenic steroids is very

evident, whereas that of "11-oxysteroid" activity is considerably less than that seen in the female subjects. Both sexes gave evidence of a sharp rebound in DCA-like steroid activity.

Metabolism during acclimatization to heat versus that observed during administration of ACTH

As indicated earlier in this report, the initial purpose of this study was to determine whether or not administration of ACTH would result in a decrease in the concentration of sodium and chloride of sweat. We had reported (2) (4) that the process of acclimatization to heat is characterized by a typical pattern of metabolism, namely, negative nitrogen balance despite adequate intake of protein, a sharp decrease of urinary excretion of sodium and chloride, an increase of urinary potassium, and rapidly falling concentrations of sodium and chloride of sweat. Also described was the finding that an increase (rebound) in urinary excretion of sodium and chloride occurred on the third to fifth day of acclimatization to heat but that the concentrations of these elements in sweat remained depressed. We had discovered subsequently (17) that urinary excretion of uric acid was greatly increased during acclimatization to heat. It was suggested that the process of acclimatization to heat consists of adrenal cortical stimulation via pituitary ACTH.

It is clearly evident (Figs. 2, 4, 6 and 8) that all of these metabolic phenomena are produced in normal people by administration of ACTH. The conclusions seem justified, therefore, that i) acclimatization to heat is an adrenocortical phenomenon mediated through increased production of ACTH and ii) the concentrations of sodium and chloride in sweat reflect changes in adrenal production of DCA-like steroid hormones.

COMMENT

The ability to observe changes in activity of "salt-active" corticosteroids is of value in clinical diagnosis (12). Of somewhat greater significance may be its potential value in investigative medicine, in elucidating the possible role of the "salt-active" corticosteroids in a wide variety of clinical conditions (*e.g.*, nephrosis, congestive heart failure, essential hypertension, premenstrual edema) in addition to the so-called "diseases of adaptation" (18).

Preliminary clinical application indicates a high degree of sensitivity of the "sweat test" in such conditions as Addison's disease, carcinoma of the adrenal cortex, Cushing's syndrome without carcinoma, and panhypopituitarism. It is helpful, too, in evaluating the adequacy of control of electrolyte metabolism during therapy for adrenal cortical insufficiency. In addition, a variety of clinical states, suspected and unsuspected of being associated with increased adrenal production of "salt-active" steroids, can

be investigated. The present two-hour-heat-exposure technic is difficult for the patient. A simplified, standardized procedure is being studied.

A considerable range of variation exists in normal people with respect to the concentrations of sodium and chloride in sweat. We believe that this variation parallels the normal variation in functional activity of the adrenal cortices among healthy individuals. In support of this belief is the fact that normal persons with low baseline values for these elements in sweat respond with much more intense metabolic changes to ACTH than do individuals with high initial values.

In the past we have studied the changing metabolism of normal men accustomed to a temperate climate, who were placed in a simulated tropical environment for a period of many weeks. Such men adapt themselves to the new climatic conditions, the process having been called "acclimatization to heat." The metabolic data indicated that the process was accomplished by virtue of increased adrenal cortical activity. Among other phenomena observed, it was found that the concentration of sodium and of chloride of sweat diminished greatly. It was suggested that the mechanism of acclimatization to heat consisted of increased production of pituitary ACTH with a resultant increase in adrenal cortical activity.

The present experiments sustain this concept. All of the metabolic changes observable during acclimatization to heat (including the persistent depression of the electrolyte concentration of sweat) can be duplicated in normal individuals during administration of pituitary adrenocorticotropic hormone.

CONCLUSIONS

1. Under standard conditions of testing, the electrolyte composition of thermal sweat affords a constant index of adrenal production of DCA-like corticosteroids.

2. The electrolyte composition of the urine (in the absence of renal disease) may be a reliable index of *deficiency* of "salt-active" corticoids but it is a poor indicator of excessive activity of these steroids because of the well known renal "rebound" phenomenon.

3. Application of the sweat test in clinical conditions involving abnormal adrenocortical function has given useful information in diagnosis and in the evaluation of therapy in deficiency states. It is believed that it may constitute a helpful tool in assessing the role of the adrenal in a variety of clinical states in which excessive production of DCA-like corticoids may be a factor.

4. Adrenocorticotropic hormone is capable of increasing adrenal elaboration of "11-oxysteroids," androgenic steroids, and DCA-like steroids in normal people.

5. After sustained administration of ACTH to normal humans, cessation of such treatment results in (a) temporary adrenocortical insufficiency, fol-

lowed by (b) temporary adrenocortical hyperfunction, followed by (c) return to normal function. Temporary depression of endogenous ACTH during administration of the exogenous hormone probably accounts for this series of effects.

6. The process by which normal men acclimatize to heat consists of increased adrenal cortical function mediated via increased pituitary production of ACTH.

REFERENCES

1. Conn, J. W., and Johnston, M. W.: Salt economy of the sweat glands under conditions of hard work in a tropical climate, *J. Clin. Investigation* **23**: 933, 1944.
2. Conn, J. W.: The Mechanism of Acclimatization to Heat. Advances in Internal Medicine, New York, Interscience Publishers, Inc., 1949.
3. Conn, J. W.; Johnston, M. W., and Louis, L. H.: Acclimatization to humid heat. A function of adrenal cortical activity, *J. Clin. Investigation* **25**: 912, 1946.
4. Conn, J. W.; Louis, L. H., and Johnston, M. W.: The electrolyte content of thermal sweat as an index of adrenal cortical function, *J. Clin. Investigation* **27**: 529, 1948.
5. Butler, A. M., and Tuthill, E: An application of the uranyl zinc acetate method for determination of sodium in biological material, *J. Biol. Chem.* **93**: 171, 1931.
6. Harvey, S. C.: The quantitative determination of chlorides in the urine, *Arch. Int. Med.* **6**: 12, 1910.
7. Shohl, A. T., and Bennett, H. B.: Determination of potassium in biological fluids, *J. Biol. Chem.* **98**: 643, 1928.
8. Benedict, S. R., and Franke, E.: A method for the direct determination of uric acid in urine, *J. Biol. Chem.* **52**: 387, 1922.
9. Buchanan, O. H.; Block, W. D., and Christman, A. A.; The metabolism of the methylated purines. I. The enzymatic determination of urinary uric acid, *J. Biol. Chem.* **157**: 189, 1945.
10. Benedict, S. R.: The detection and estimation of glucose in urine, *J.A.M.A.*, **57**: 1193, 1911.
11. Robbie, W. A., and Gibson, R. B.: Rapid clinical determination of urinary 17-ketosteroids, *J. Clin. Endocrinol.* **3**: 200, 1943.
12. Conn, J. W.: Clinical implications of the electrolyte composition of sweat as an index of adrenal cortical function, *Arch. Int. Med.* **83**: 416, 1949.
13. Forsham, P. H.; Flink, E.; Emerson, K., Jr., and Thorn, G. W.: Metabolic studies on Cushing's syndrome, (abstract) *J. Clin. Investigation* **28**: 781, 1949.
14. Conn, J. W.; Louis, L. H., and Wheeler, C. E.: Production of temporary diabetes mellitus in man with pituitary adrenocorticotropic hormone; relation to uric acid metabolism, *J. Lab. & Clin. Med.* **33**: 651, 1948.
15. Conn, J. W.; Louis, L. H., and Johnston, M. W.: Metabolism of uric acid, glutathione and nitrogen and excretion of "11 oxysteroids" and 17-ketosteroids during induction of diabetes in man with pituitary adrenocorticotropic hormone, *J. Lab. & Clin. Med.* **34**: 255, 1949.
16. Conn, J. W.; Louis, L. H., and Johnston, M. W.: Alleviation of experimental diabetes in man by intravenous administration of reduced glutathione, *Science* **109**: 279, 1949.
17. Louis, L. H., and Conn, J. W.: Unpublished data.
18. Selye, Hans: The general adaptation syndrome and the diseases of adaptation, *J. Clin. Endocrinol.* **6**: 117, 1946.

THE ROLE OF THE ADRENAL CORTEX IN SOMATOSEXUAL DISTURBANCES IN INFANTS AND CHILDREN: A CLINICO-PATHOLOGIC ANALYSIS*

M. M. MELICOW, M.D. AND G. F. CAHILL, M.D.

From the Squier Urological Clinic, Columbia University-Presbyterian Hospital, New York

SIGNIFICANT deviations from the relatively normal somatosexual pattern are rare in spite of the pronounced complexity in the processes of differentiation of the embryo. When deviations occur, they usually cause consternation to the parents, perplexity to the physician, and tragedy to the victim. Aberrations from the normal somatosexual make-up may be present at birth, or develop during infancy, childhood or puberty. An erroneous conclusion as to the true sex often leads to irreversible psychosomatic fixation and trauma to the patient, the degree depending on the age when the true sex is finally discovered. Some heteroclites grow up as notorious medical "cases"; others become circus curiosities; and still others carry their abnormality locked like a skeleton in their own somatosexual closet, fearful of exposure, embittered in their lot, antisocial and suicidal in their outlook.

The basic sex of an individual is apparently predetermined by the genes but definitive differentiation is not accomplished until about the sixth week of intrauterine life. Further elaboration and development, particularly of the secondary sexual apparatus, is postponed until puberty and is then under the control of the individual's own endocrine system. *True* hermaphrodism is probably genotypical in origin and should be distinguished from *pseudo*hermaphrodism which usually results from endocrine hyperfunction or dysfunction, maternal and/or fetal. The true hermaphrodite possesses adequate gonadal tissue or gonads of *both* sexes (ovario-testis, or ovary and testis); the pseudohermaphrodite is basically of one sex, but outwardly the sexual pattern resembles that of the opposite sex.

Varying degrees of pseudohermaphrodism have been produced in laboratory animals, the degree depending on the fetal age when estrogen or androgen extracts were injected into the pregnant mother (Greene, Burrill and Ivy; Hamilton and Wolfe; Turner (1,2,3)) (Table 1). If injected during the early stages of pregnancy, they caused retention of those cephalad elements

Received for publication June 8, 1949.

* Read by title at the Annual Meeting of the Association for the Study of Internal Secretions, Atlantic City, June 3 and 4, 1949.

of the opposite sex which become attenuated in normal differentiation (Müllerian anlages in males, Wolffian in females), and arrest or retardation of those characteristics that are normally defined and elaborated. Injections given later produced aberrations in the caudal end of the genital tract: males were born with abdominal or partially descended testes, cleft

TABLE 1. EFFECT OF HORMONE INJECTIONS INTO PREGNANT RATS OR MICE*

	Androgen-injected	*Estrogen-injected*
	Aberrations in female offspring, present at birth	Aberrations in male offspring, present at birth
During early stages of pregnancy (in mice: ten to fourteenth day of gestation)	1. Retention of upper Wolffian derivatives 2. Inhibition of Müllerian derivatives	1. Retention of upper Müllerian derivatives 2. Inhibition of Wolffian derivatives
During later stages of pregnancy (in mice: after fifteenth day of gestation)	1. Retention of lower Wolffian derivatives and inhibition of parts of Müllerian derivatives 2. Inhibition of parts of uterine tract 3. Retention of remnants of epididymal tract 4. Ovaries unaffected 5. Large clitoris. Female urethra 6. Large labia 7. ± Agenesis of vagina 8. ± Retention of remnants of prostate	1. Retention of lower Müllerian derivatives and inhibi- of parts of Wolffian derivatives 2. Inhibition of parts of epididymal tract 3. Retention of parts of uterine tract 4. Testes remain in abdomen 5. Small penis. Hypospadias urethra 6. Cleft scrotum 7. ± Retention of vagina 8. ± Absence of prostate

* Based on experimental work of Greene, Burrill and Ivy; Turner; and Hamilton and Wolfe (1, 2, 3).

scrotum, small penis with hypospadias, vestigial vagina; females showed enlarged clitoris, large labia, atretic vagina, and occasionally retention of prostatic elements. These experiments suggest that pseudohermaphrodism in the human may also result from disturbed or altered endocrines of maternal (4) and/or fetal origin which affect the genital primordia of the embryo at a crucial stage of differentiation (Fig. 1).

Excess androgens induce "maleness" in genetic females so that the child presents a large phallus at birth. Doubt arises as to whether it is a male with a small penis, cleft scrotum, hypospadias and abdominal testes, or a female with a large clitoris and small vagina (female pseudohermaphro-

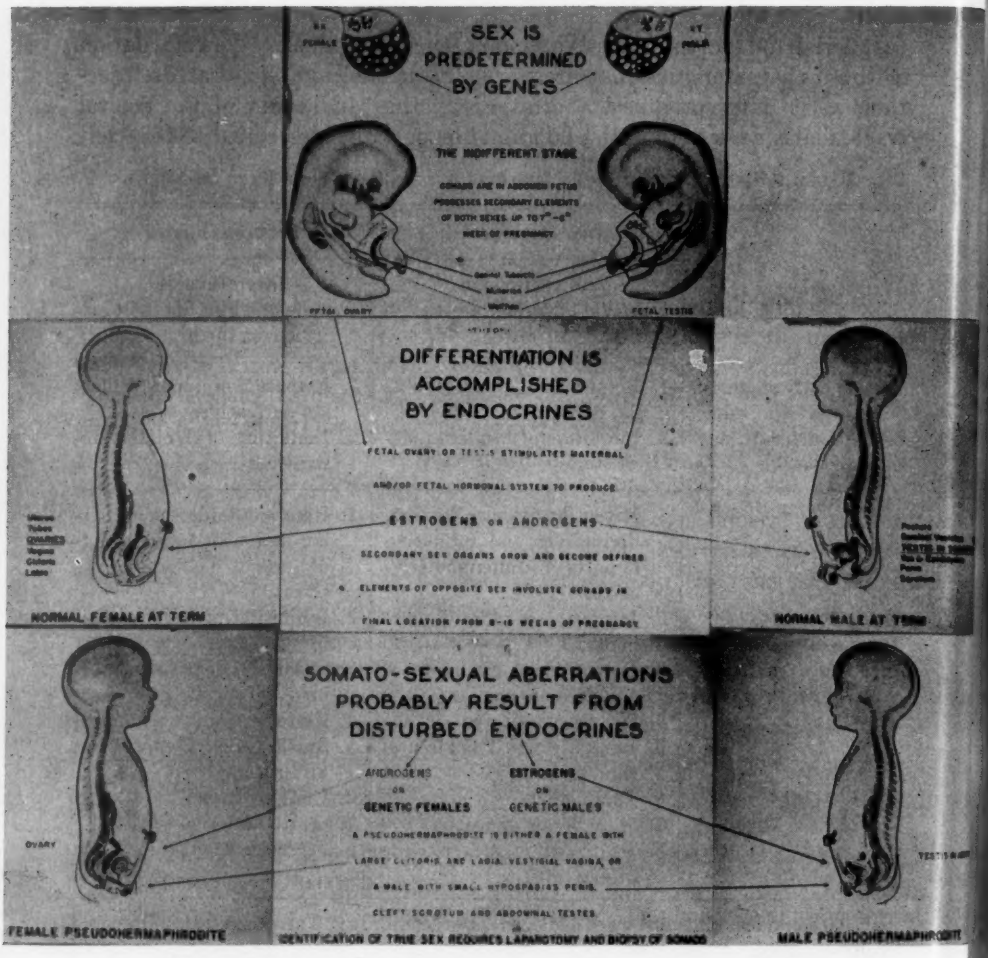

FIG. 1. Schematic representation of possible mechanism involved in the development of male and female pseudohermaphrodites (Melicow).

dite). The effect of estrogenic overfunction, or more likely dysfunction, is manifest in genetic males in whom it induces "femaleness" (male pseudohermaphrodite). Here, too, the problem is one of ascertaining the true sex.

After birth, alteration in the normal somatosexual pattern may result from hyperfunction, hypofunction, or dysfunction of one or more glands in the endocrine chain: pituitary, pineal, thyroid, thymus, gonads, and adrenals (5, 6, 7, 8). Our increased knowledge of the functions of the adrenals has enabled us to recognize that a large group of somatosexual abnor-

malities result from a disturbance emanating from its cortex. This structure deve'ops from the gonadoplasmic mesoderm wh'ch is capable of elaborating androgens and estrogens. The clinical picture induced by cortical hyperfunction depends on the *age* and *sex* of the patient, and on whether the excess is *androgenic* or *estrogenic*. Androgen hyperfunction in childhood tends to accelerate the onset of puberty, maleness being superimposed on genetic females and accentuated in genetic males—the adrenogenital or corticosexual syndrome (9, 10). Estrogen hyperfunction is relatively rare and presents an opposite picture. Table 2 illustrates the effect of the two types of cortical hyperfunction.

TABLE 2. RELATION OF SOMATOSEXUAL ABERRATIONS TO 1) TYPE OF ENDOCRINE DISTURBANCE, 2) SEX, 3) AGE (MELICOW).

	EXCESS ANDROGENS		EXCESS ESTROGENS	
	MALE	FEMALE	MALE	FEMALE
	Maleness accelerated and exaggerated	Maleness superimposed on basic female	Femaleness superimposed on basic male	Femaleness accelerated and exaggerated
**Intrauterine* (noted at or soon after birth)	*Macrogenitosomia (male)* Born with large penis and well-developed scrotum	*Pseudohermaphrodism (sex in doubt)* Born with large clitoris, large labia, small vagina	*Pseudohermaphrodism (sex in doubt)* Born with small penis, hypospadias, cleft scrotum, undescended testes	*Macrogenitosomia (female)* Born with large breasts, ± vaginal bleeding, ± obesity
	The Corticosexual (Adrenogenital) Syndrome			
During infancy and childhood	†*Precocious Puberty ("child Hercules")* Penis and scrotum enlarged, tall for age, hirsutism, coarse voice, muscular	*Masculinization ("tomboy")* Clitoris enlarged, tall for age, hirsutism, male pubic hair line	*Feminization ("girlish boy")* Penis decreased in size, testes small, straight pubic hair line, gynecomastia	*Precocious Puberty ("womanly girl")* Unusually large breasts, curvaceous figure, menses, female pubic hair line, ± obesity
At puberty	†*Precocious Male ("virilism")* Adult penis and scrotum, hirsutism, adult male voice. Epiphyses fused and growth stopped. If not halted, "adrenal dwarf"	*Masculinization ("virilism")* Oligo- or amenorrhea, clitoris enlarged, hirsutism, male pubic hair line, coarse voice, narrow hips, athletic build	*Feminization ("girlish male")* Gynecomastia, penis and testis small, straight pubic hair line, wide hips, feminine voice	‡*Precocious Female ("matronly girl")* Unusually large breasts, curvaceous figure, menses, female pubic hair, ± obesity

* Cortical hyperfunction, except in the case of neoplasia, is usually not demonstrable at birth; some of our patients showed it later. Experimental evidence suggests it.

† Precocious puberty in males can also be the result of hyperfunction with or without neoplasia of the pineal, of the anterior pituitary, or of the interstitial cells of the testis.

‡ Precocious puberty in females can also be the result of hyperfunction with or without neoplasia of the anterior pituitary, or of the ovary.

Cortical hyperfunction may be self-limited or indefinitely prolonged, and may or may not be associated with hyperplasia or neoplasia, benign or malignant, unilateral or bilateral. It follows that: 1) a certain number of patients with somatosexual abnormalities will no longer reveal evidence of

cortical hyperfunction by the time they seek medical aid; 2) a considerable number will show no adrenal enlargement in spite of evidence of overactivity, as indicated by clinical and laboratory studies; 3) a fairly large number will show unilateral or bilateral diffuse adrenal enlargement; and 4) in some cases the adrenal gland may be the seat of a neoplasm, benign or malignant.

The term "somatosexual pattern" refers not only to the appearance of the external genitalia, but also to the status of the internal and secondary sex organs. It takes cognizance of the general body contour, skin texture, presence and distribution of hair, quality of voice, muscle build, bone age, and urine 17-ketosteroid content.

Experience has taught us that the determination of true sex in a pseudohermaphrodite and correction of the deformity should be carried out before psychosomatosexual distortions have become fixed and irreversible. This is a *process* which may extend over a period of *weeks, months or years*. It was necessary in many cases to resort to laparotomy and biopsy of the gonads. The following are some of the procedures that were carried out in most of our cases:

1. Detailed history of the case, of significant disorders in other members of the family, and of the mother's condition during pregnancy, noting any endocrine disturbance suffered by her during pregnancy, or the taking of hormones orally or by injection.
2. Physical examination, paying particular attention to height, weight, body contour, hair distribution, muscularity, size of breasts, and, in the external genitalia, to the size of the phallus, presence of erections, location of urethra, location and size of "vagina," size of labia or scrotum, and presence of a mass in the labia. Rectal examination, in order to note the presence and size of the prostate, or of uterus and cervix. Kodachromes, black and white photographs of the body as a whole, and a close-up of genitalia and other significant areas, followed by interval photographs.
3. Studies of the endocrine system, including radiographs of the pineal gland and sella turcica, basal metabolism, hormonal assays (when feasible), and urinary 17-ketosteroids. Blood chemistry and study of metabolites (11, 12, 13).
4. Determination of bone age as indicated by radiographic studies.
5. Radiographs and aerograms of the adrenal region (14, 15). Intravenous pyelogram studies. Cystoscopic examination and Diodrast studies of abnormal channels and diverticuli. Retrograde pyelograms when indicated.
6. Laparotomy and biopsy of gonads.

Treatment depended on findings and was carried out according to the following plan:

A. In cases *without* demonstrable disturbance in the adrenal glands:
   1. Clitoridectomy in pseudohermaphrodites that were proven to be females.
   2. Construction of a scrotal sac, repair of hypospadias and orchidopexy in pseudohermaphrodites that were proven to be male.

B. In cases *with* demonstrable disturbance in one or both adrenal glands:
   1. Resection of part of one or of both adrenal glands when shown to be enlarged. Removal of tumor when present (16).
   2. Clitoridectomy in females if recession does not occur in spite of above procedure, or when the enlargement of the clitoris is unduly pronounced.
   3. Plastic repair in males, as discussed under "A."

C. Psychotherapy of patient and parents, particularly in cases where error in sex had been made. Management of infants or young children is relatively easy, but is somewhat difficult in older children. The parents are usually emotionally disturbed, and need enlightenment and advice. A change of environment is desirable.

D. Hormonal therapy, depending upon the type of disturbance.

Our cases fell mainly into two of the following four groups as shown in Table 3:

Group I: Somatosexual abnormalities in infants and children *without demonstrable adrenocortical enlargement* but with suggestive clinical and laboratory evidence of cortical androgenic hyperfunction.

Group II: Somatosexual abnormalities in infants and children *with demonstrable adrenocortical enlargement or tumor* as well as clinical and laboratory evidence of cortical androgenic hyperfunction.

Group III: Male pseudohermaphrodism with no evidence of active adrenal androgenic or estrogenic hyperfunction.

Group IV: Cushing's syndrome.

GROUP I

Somatosexual abnormalities in infants and children ***without*** *demonstrable adrenocortical enlargement but with suggestive clinical and laboratory evidence of cortical androgenic hyperfunction.*

A. In genetic females

There were 4 cases in this group. Three gave clinical and laboratory evidence suggestive of cortical hyperfunction, but none showed enlargement of the adrenal gland by radiographic studies or aerograms. All were

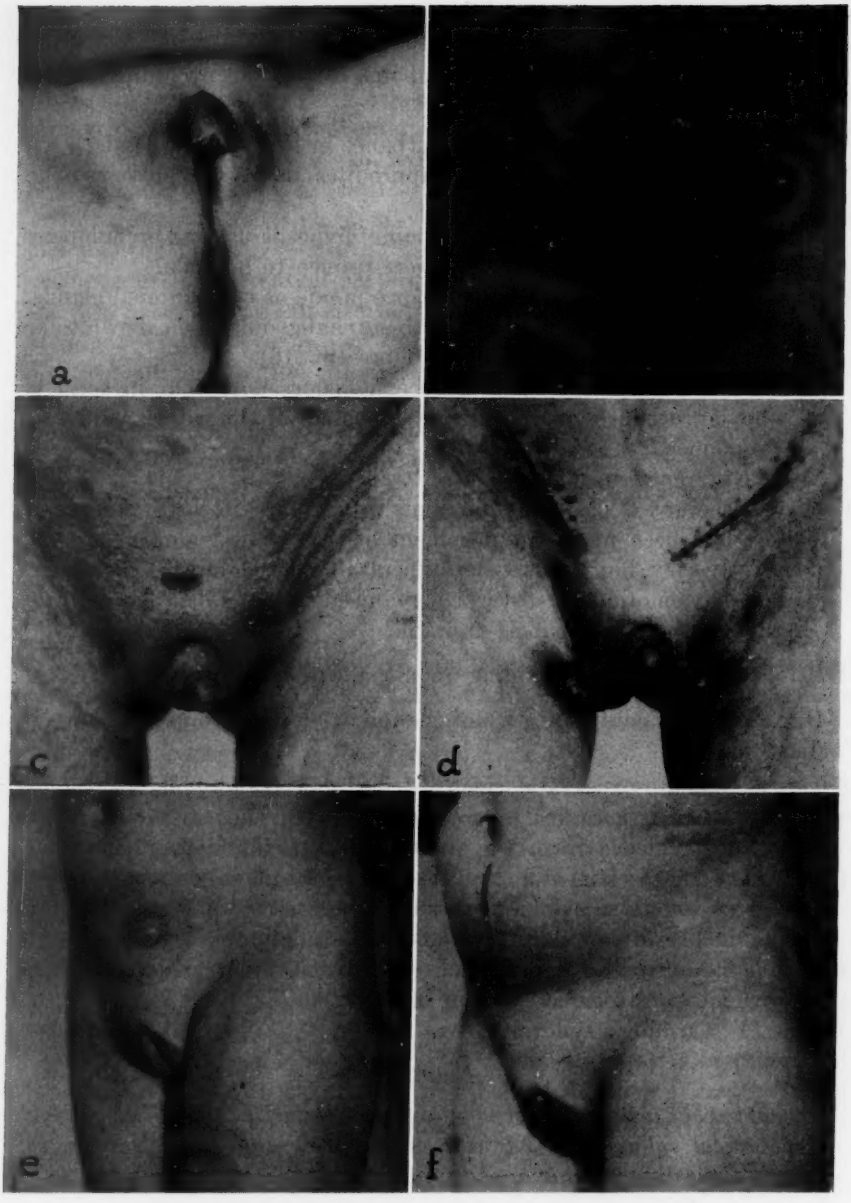

FIG. 2. *a*) Case 1, J. B., an 11-day-old infant with a large clitoris. Sex in doubt, until laparotomy and biopsy of gonads (Dr. Cahill) at 20 months of age established true sex of child (female). *b*) Case 16, J. K., age 3 years, a male with cleft scrotum and Müllerian remnants. *c & d*) Case 17, E. P., age 6 years, a child whose sex (male) was established by laparotomy and in whom later orchidopexies (Drs. Yeaw, Robinson, Barrett) were performed. *e & f*) Case 7, J. L., age 4 years, with a corticosexual syndrome and enlarged clitoris; before and after laparotomy and clitoridectomy (Dr. Cahill).

pseudohermaphrodites. In 2, there was doubt as to the true sex from birth (Cases 1 and 2); the other 2 (Cases 3 and 4) were erroneously raised as males with supposed undescended testes. In the first 2 cases the ages on admission were 11 days and 20 months, respectively; in the latter 2 (who were looked upon as males), 4 and 4½ years, respectively. Exploratory laparotomy in 3 of the cases revealed a uterus, tubes, and ovaries, proven by biopsy. Clitoridectomy was then performed, and the 2 children that had been brought up as boys had their names changed. The families were advised to move to new surroundings. The presence of a uterus and cervix was indicated by rectal examination in 1 of the cases. This patient is still being studied. Some of the infants revealed a relative increase in urinary 17-ketosteroids, and the majority (Cases 2, 3, and 4) showed accelerated ossification.

Case 1, J. B., 757920, was admitted on the eleventh day after birth because of doubt as to true sex. The clitoris was enlarged, the urethral opening was at its base, and the labia were much enlarged (Fig. 2a). The urinary 17-ketosteroids were 2.0 mg./24 hrs. The patient was readmitted twenty months later, at which time laparotomy (Dr. Cahill) was performed, and a uterus, tubes and ovaries (biopsy) were found. The adrenals were apparently normal. The child being a female, clitoridectomy (Dr. Cahill) was therefore performed. Radiographs of hands and forearms indicated bone age characteristic of a 6-year-old child. The patient returned at the age of 3 years with pubic hair and hair on the vulva. Urinary 17-ketosteroids rose to 7.6 mg./24 hrs. She is to return for further studies. *Conclusion*: A female child whose feminine characteristics were being submerged by an androgenic influence. Exploratory laparotomy and biopsy of the gonads were necessary to establish the true sex. The androgenic overactivity probably emanated from a hyperfunctioning adrenal cortex (corticosexual syndrome).

Case 2, T. T., 870462, was a 20-month-old child admitted to the Urologic Service of Babies Hospital because of a large clitoris present since birth. The mother was in doubt as to the child's true sex. Examination revealed a normal-looking female child. A hooded prepuce characterized a prominent clitoris which was 2 cm. in length. There was no definite evidence of a vagina on superficial examination. However, under general anesthesia, a vagina and cervix were demonstrated, and rectally a vertical mass was felt in the region of the uterus. Urinary 17-ketosteroids were 5.3 mg./24 hrs. Aerograms of the adrenals were indefinite; these were repeated four months later and again showed no distinct pathology. The child was readmitted again at the age of 2½ years and aerograms were repeated. There was a suspicion of the right adrenal being enlarged. Laparotomy (Dr. Cahill) revealed a uterus, tubes and ovaries. Clitoridectomy was then performed. *Conclusion:* A child, small for her age, with enlarged clitoris and probably a female. Adrenocortical hyperfunction was suggested by elevated urinary 17-ketosteroids and by the aerograms. Patient will return for further study.

Case 3, T. K., 674516. The patient, raised as a boy, was admitted at the age of 4 years because of supposed undescended testes, enlarged phallus and pubic hair, since the age of 2 years. Examination revealed a well-developed child with a "penis" 7 cm. long and with moderate growth of pubic hair. The scrotum was cleft and resembled labia majora. At the root of the "penis," between the two labia was the urethra. No testes were palpated. Urinary 17-ketosteroids were 26 mg./24 hrs. Roentgenograms of the skeleton showed a

TABLE 3. PSEUDOHERMAPHRODISM

GROUP I. *No demonstrable adrenal enlargement, but suggestive clinical and laboratory evidence of cortical androgenic hyperfunction.*

Case No.	Initials	Age when first seen	History	Appearance	Adrenals	
					Roentgenogram	Aerogram
1	J.B.	11 days	Parents and physician in doubt as to true sex, from birth.	Large hypospadic phallus and "labia." No vaginal orifice. No testes in "labia."	Neg.	—
2	T.T.	20 months	Parents in doubt as to true sex.	Same as above.	Neg.	—
3	T.K.	4 years	Raised as boy with supposed undescended testes.	"Penis" and pubic hair, cleft "scrotum," no testes. "Hypospadias."	Neg.	Neg.
4	R.K.	4½ years	Raised as boy until exploratory laparotomy showed uterus, tubes and ovaries.	"Penis" and bifid "scrotum." No testes. Pubic hair. "Hypospadias."	Neg.	Neg.
5	L.S.	11 years	Macrogenitosomia since age of 2 years. Tall at 7, stationary since.	Adult male. Coarse beard (shaved daily), and male voice. Cigar smoker. Large penis. Muscular.	?	Neg.

GROUP II. *Demonstrable adrenal enlargement or tumor as well as suggestive clinical and laboratory evidence of cortical androgenic hyperfunction.*

6	K.A.	3½ years	Raised as female but doubt developed when phallus enlarged.	Large phallus, "female" urethra, and "labia." Pubic hair. Muscular.	At 3½ years, neg.	At 6 years—left adrenal enlarged At 7 years—right adrenal enlarged
7	J.L.	4 years	Raised as girl but sex in doubt for several years due to change in genitalia.	Acne, male habitus, large phallus.	Neg.	At 4 years—neg. At 8 years—left adrenal enlarged
8	M.H.	7 years	Gradual alteration of female child toward maleness (clitoris enlarged)	Tall and hirsute. Deep voice and large clitoris.	Mass in region of rt. adrenal	Suggestive of tumor of right adrenal
9	E.R.S.	7 years	Same as preceding patient.	Acne, large clitoris, deep voice, hirsutism. Clitoridectomy performed elsewhere.	Mass above left kidney	Enlarged left adrenal
10	C.A.	7	Typical female until five months before admission, when hirsutism and male voice developed.	Large clitoris. Acne. Marked hirsutism.	Mass above right kidney	Same
11	C.A.	7 years	Patient always looked like boy. Had 5 operations for supposed hypospadias. Treated for supposed undescended testes.	Large "penis." Diffuse hirsutism, male habitus and coarse voice. Scoliosis. No palpable prostate.	—	Bilateral enlargement
12	M.M.	12 years	Raised as female to age of 6 years when physician suspected that enlarging phallus was a penis.	Large phallus. Hirsutism. Male pubic hair line.	Enlarged left adrenal	Same
13	A.R.L.	2 years	Macrogenitosomia since birth.	Large penis and adult scrotum. Deep voice. Strong and muscular.	Enlarged right adrenal	Same
14	R.H.	4½ years	Gradual development of large penis.	Acne. Hirsutism.	?	Enlarged left adrenal
15	F.H.	6 years	Gradual development of macrogenitosomia.	Aggressive. Acne, large penis, erections, male pubic hair line, coarse voice.	Enlarged left adrenal	Tumor of left adrenal

Urinary 17-ketosteroids (mg./24 hrs.)	Bone maturation	Operation	Conclusion
At 11 days—2.0 At 3 years—7.6	At 21 months:—6 years	Laparotomy: uterus, tubes, ovaries (biopsy). Clitoridectomy.	Genetic *female*.
5.3	—	Laparotomy: uterus, tubes, ovaries (biopsy). Clitoridectomy.	Probably genetic *female*.
At 4 years—26 At 5 years—9.2 At 6 years—22.8	At 4 years:—12 years At 5 years:—16 years	Laparotomy: uterus, tubes, ovaries (biopsy). Clitoridectomy.	Genetic *female*. Name changed.
9.8	—	Laparotomy: uterus, tubes, ovaries (biopsy). Clitoridectomy.	Genetic *female*. Name changed.
—	Advanced ossification. At 11 years:—40 years	None	Male "adrenal dwarf."
At 3½ years—18.9 At 6 years—14.8 At 7½ years—35.2 At 12 years—43.2	At 3½ years:—8 years At 7 years:—12 years	Laparotomy at 3½ years: uterus, tubes, ovaries (biopsy). At 6 years partial excision left adrenal. At 7½ years: same; right adrenal (granular cell hyperplasia). Clitoridectomy.	Masculinization in a genetic *female* due to cortical hyperfunction.
At 4 years—14.8 At 8 years—39.1	At 4 years:—7 years At 8 years:—17 years	Laparotomy at 4 years: uterus, tubes, ovaries (biopsy). No operation on adrenals. Clitoridectomy.	Masculinization in a genetic *female* due to cortical hyperfunction.
Preoperative: 173.0 Postoperative: 2.6	Tall for age	Granular cell adenoma. Right adrenalectomy.	"Boyish girl." Clitoris receded.
16.4	Tall for age	Adenoma of adrenal cortex; ganglioneuroma. Tumor excised.	"Boyish girl." Recession after operation.
76.8	At 7 years:—12 years	Inoperable granular cell carcinoma of adrenal.	Died seven days after operation.
At 7 years—42.8 At 10 years—56.7 At 12 years—64.8	At 7 years:—25 years	Laparotomy: uterus, tubes, ovaries (biopsy). No testes, but Marchand bodies. Bilateral partial excision of adrenals for granular cell hyperplasia. Hysterectomy and oophorectomy.	Genetic *female*, somatically male in appearance due to profound cortical androgenic overactivity.
43	At 12 years:—20 years	Laparotomy: uterus, tubes, ovaries (biopsy). Partial excision of left adrenal for granular cell hyperplasia. Clitoridectomy.	Masculinization in a genetic *female* due to cortical hyperfunction.
16.2	At 2 years:—8 years	Partial excision of right adrenal for granular cell hyperplasia.	"Child Hercules."
18.8	At 4 years:—13½ years	None.	"Child Hercules."
Preoperative: 189 Postoperative (1 year later): 5	At 6 years:—14 years	Granular cell adenoma of left adrenal cortex. Encapsulated tumor, weighing 147 Gm., removed.	"Precocious puberty."

Case No.	Initials	Age when first seen	History	Appearance	Adrenals	
					Roentgenogram	Aerogram
GROUP III. *Male pseudohermaphrodism with no evidence of active adrenal androgenic or estrogenic hyperfunction.*						
16	J.K.	3 years	Raised as female but parents not sure about sex for last two years.	Large hypospadic phallus, small vaginal orifice.	Neg.	—
17	E.P.	6 years	Parents were told baby was a girl at birth.	Hypospadic phallus. Vagina present (colpogram). Cleft scrotum.	Neg.	Neg.
18	S.S.	10 years	Raised as female, but recently mother noted swelling in "labia."	Female habitus. Small phallus, hypospadias. Mass in left labium.	Neg.	—
19	R.F.	17 years	Raised as female but no menses. Voice became coarse. Mother suspicious.	Male habitus. Hirsutism. Penis-like phallus. Hypospadias.	Neg.	Neg.
GROUP IV. *Cushing's syndrome*						
20	A.G.	11 years	Gradual development of moon face, trunk obesity, fatiguability and hirsutism.	Characteristic picture of Cushing's syndrome.	Enlarged right adrenal	Mass in region of right adrenal

bone age of 12 years. Exploratory laparotomy (Dr. Cahill) was performed, revealing a uterus, tubes and gonads which proved to be ovaries on biopsy. It was therefore evident that this child was a female erroneously brought up as a male. Clitoridectomy (Dr. Cahill) was then performed. The patient was given a feminine name, and estrogenic hormones were administered. After operation, she presented a feminine habitus. However, there was no recession of accelerated bone development (Fig. 3 a, b, c, d). *Conclusion:* A female brought up as a boy with undescended testes, until laparotomy and biopsy of gonads revealed the true sex. The corticosexual syndrome is indicated by the elevated urinary 17-ketosteroids and the accelerated bone development.

Case 4, R. K., 884664, was a 4½-year-old child brought up as a male because of a large "penis" and bifid "scrotum." However, one year before admission to the Urologic Service of Babies Hospital, an exploratory laparotomy had been performed at Johns Hopkins Hospital, and a normal-looking uterus, tubes and ovaries were found. Since then, pubic hair had appeared. Examination revealed a clitoris 3 cm. long with redundant prepuce, large labia and female type of urethra. No vaginal opening could be seen. Urinary 17-ketosteroids were 9.8 mg./24 hrs. Aerograms of the adrenals revealed no enlargement. Clitoridectomy (Dr. Cahill) was performed, and the child given a female name. *Conclusion:* A 4½-year-old girl erroneously brought up as a boy because of a large clitoris. The underlying condition was probably an adrenocortical hyperfunction (corticosexual syndrome).

B. In genetic males

One of our patients (Case 5) probably belongs in this category. The patient was an 11-year-old boy with pronounced clinical evidence of cortical hyperfunction. He showed great muscular development; his bone age

Urinary 17-ketosteroids (mg./24 hrs.)	Bone maturation	Operation	Conclusion
1.6	Normal	Laparotomy: *no* uterus, tubes, or ovaries. Right testis (biopsy) in inguinal canal. No vas. No testis on left side. Müllerian remnant and vaginal epithelium found. Plastic operation on penis and scrotum. Orchidopexy.	Genetic *male*, with Müllerian remnants.
1.2	Normal	Laparotomy: *no* uterus, tubes, or ovaries. Bilateral masses in groin (biopsy: testes). False vagina extirpated. Repair of hypospadias and chordee.	Genetic *male*.
—	Normal	Laparotomy: *no* uterus, tubes, or ovaries. Vas on each side leading to undescended testes biopsy). Orchidopexy.	Genetic *male*. Name changed.
6.4	Normal	Laparotomy: uterus and tubes present, but gonads proved to be *testes*. Hysterectomy. Orchidopexy.	Genetic *male*. Name changed.
	Normal. Osteoporosis.	Exploratory, of right adrenal.	Death due to severe infection of wound.

was that of a 40-year-old man; he had an adult male voice, was quite hairy and shaved daily; the penis was large, and the scrotum was of adult type. Yet the results of laboratory and radiographic studies, including aerograms of the adrenals, were negative. We suspected that this boy's adrenal cortex had been the seat of an extreme degree of overactivity which, however was self-limited, and that we were dealing with an irreversible end-process. Earlier, the boy was probably tall for his age but after his epiphyses joined, his stature became static, resulting in an "adrenal dwarf."

Case 5, L. S., 379627. This patient was admitted to Babies Hospital at the age of 11 years because of macrogenitosomia which had started when he was 2 years old. Hair began growing on his face and body at 5 years; on admission, it was generalized and he had to shave. He had not lost his original teeth. At the age of 7, he was tall, but since then has ceased to grow. Examination revealed a short-fingered, short-limbed, long-bodied dwarf of achondroplastic type, extremely hirsute and muscular (Fig. 4 d). His 3-day-old beard was luxuriant. The body and legs were well covered with hair of adult male distribution. The voice was deep and of the male type. The rest of the examination was essentially negative except for a large penis, scrotum and testes. His blood pressure was 112/60, weight 41 Kg., and height 135 cm. During his stay in the hospital, he smoked cigars daily with considerable relish. Roentgenograms of the skeleton revealed unusually advanced epiphyseal development with considerable lateral bowing of the legs. Aerograms showed no evidence of adrenal tumor. His IQ was 69. *Conclusion:* A male child who went from the stage of "child Hercules" to "adrenal dwarfism" possibly because of an unrecognized, uninterrupted cortical hyperfunction.

FIG. 3. *a, b, c, d)* Case 3, T. K., age 4 years, and later at 6 years; and *e & f)* Case 11, C. A., age 7 years.

The 2 cases, both females, illustrate the masculinizing effects of cortical hyperfunction. Photos *a & c* show the child at the age of 2 years, erroneously raised as a boy with

FIG. 4. *a*) Case 13, A. R. L., age 2 years. *b*) Case 14, R. H., age 4½ years. *c*) Case 15, F. H., age 6 years. *d*) Case 5, L. S., age 11 years.

The series illustrates the progressive stages of precocious androgenous somatosexual differentiation in boys caused by cortical hyperfunction associated with hyperplasia or neoplasia, unilateral or bilateral. The youngest boy, aged 2 years (photo *a*) is tall for his age, and is a "child Hercules." The oldest, aged 11 (photo *d*) has "burned out" and is now short for his age. His epiphyses have joined and he has become an "adrenal dwarf." The latter illustrates the end result of unmitigated cortical androgenous overactivity in the young. In Case 14 (photo *b*), a boy aged 4½ years, the urinary 17-ketosteroids were elevated but the aerograms did not reveal any enlargement of the adrenal glands. The patient responded well to estrogenic therapy. In Case 15 (photo *c*), a boy aged 6, the aerograms revealed a mass in the region of the left adrenal, and the urinary 17-ketosteroids were 189.0 mg./24 hrs. Improvement followed the removal (Dr. Cahill) of an adenoma of the left adrenal cortex weighing 147 Gm.

---

penis and undescended testes. Laparotomy (Dr. Cahill) revealed a uterus and ovaries. Photo *b* shows the same child at the age of 6 with long hair and the effects of estrogenic therapy. Photo *d* shows the appearance of the vulva after clitoridectomy. Photo *e* shows a female aged 7 in whom the androgenic effects were so great that the child was raised as a male. Photo *f* shows the hypospadic "penis" (really a large clitoris and female urethra). The somatosexual aberration was so pronounced and so well-established that it had become irreversible. It was therefore thought advisable to remove the uterus and ovaries. The patient's adrenals were enlarged, and Marchand bodies were found in the broad ligament.

GROUP II

Somatosexual abnormalities in infants and children ***with*** *demonstrable adrenocortical enlargement or tumor as well as clinical and laboratory evidence of cortical androgenic hyperfunction.*

A. In genetic females

There were 7 cases in this group. All presented not only clinical and laboratory evidence of cortical hyperfunction, but also operative and his-

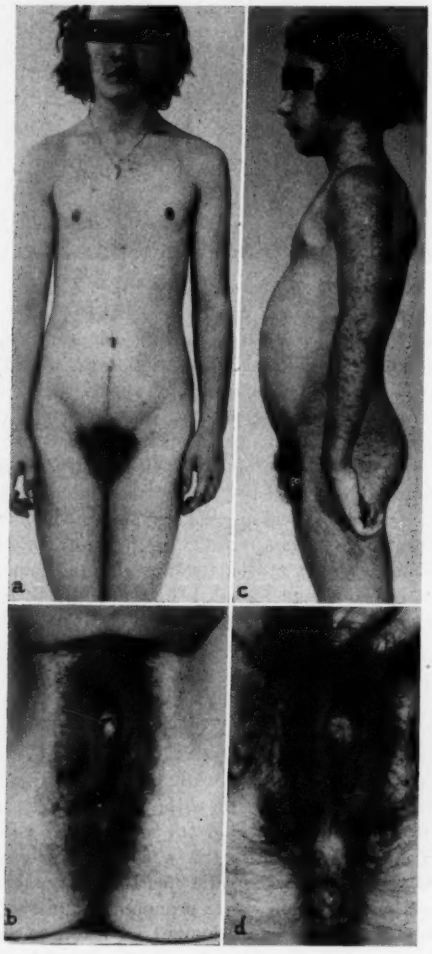

FIG. 5. Corticosexual syndrome in 2 females, one with a cortical adenoma (*a* & *b*, Case 8, M. H., age 7) and the other with a carcinoma (*c* & *d*, Case 10, C. A., age 7). Note hirsutism, acne, and enlargement of clitoris.

tologic proof of enlargement or tumor. All were females, and each possessed a uterus, tubes and ovaries. Their ages were 3½, 4 (four cases), 7, and 12 years. One of the patients, aged 7 (Case 11), was brought up as a boy. Five operations had been performed elsewhere to correct a supposed hypospadias. Our study revealed the presence of a uterus, tubes and ovaries, so that the child was genetically a female. Both adrenal glands were enlarged, and the broad ligament contained accessory cortical tissue (Marchand's adrenals). The intense and prolonged androgenic drive had remodeled this female so that she looked like a postpubescent male with a coarse voice, generalized hairiness, good muscular build, and large phallus. The altered status was now irreversible, so it was deemed wise to remove the uterus and ovaries. In spite of partial resection of both adrenals and removal of the Marchand body, this neutral individual is still under the influence of large quantities of androgen, as indicated by the high concentration of its degradation products in the urine (urinary 17-ketosteroids, 65 mg./24 hrs.). Two of the patients (Cases 8 and 9) had adrenocortical adenomas and made excellent postoperative recovery. One child, a 7-year-old girl (Case 10) had carcinoma and died. The adrenals in the remaining cases were hyperplastic (unilateral in Cases 7 and 12, and bilateral in Cases 6 and 11). Very high urinary 17-ketosteroid levels were found in the patients with tumor (Case 8—173 mg./24 hrs.; Case 10—76.8 mg./24 hrs.). Clitoridectomy was performed in Cases 6, 7, 9, and 12. In Case 8 there was recession in the size of the clitoris following removal of the cortical adenoma. The microscopic picture in all the cases revealed a preponderance of granular cells resembling those of the reticular zone (Fig. 9 a and b). "Foam cells" were occasionally present but were smaller and fewer than those seen in specimens removed from patients with Cushing's (corticometabolic) syndrome.

Case 6, K. A., 581931. The patient was brought to the hospital at the age of 3½ years because of a large phallus which had been present since birth. The family physician had declared that the child was a boy and it was brought up as one. Six months prior to admission, pubic hair was noted. Examination revealed a girlish looking "boy." The supposed scrotum was composed of large prominent labia devoid of gonads. The phallus was 2 inches in length and did not contain a urethra. The latter was located below the base of the phallus, the normal site for a female urethra. A small vaginal outlet was seen. Roentgenograms of the left wrist and knee showed markedly accelerated maturation; the sella turcica was normal. Aerograms revealed some enlargement of both adrenals (Fig. 7 b). Laparotomy (Dr. Cahill) revealed a uterus, tubes and ovaries (proven by biopsy). The clitoris was then amputated and the child re-named. However, she continued to grow rapidly and to show evidence of masculinization. Her clitoris again enlarged and the urinary 17-ketosteroids rose to 20.4 mg./24 hrs. Three years after her first admission, partial excision of an enlarged left adrenal was done. Later, because of continued accelerated maturation and masculinization (urinary 17-ketosteroids rose to 28.3 mg./24 hrs.), exploration and partial excision of the right enlarged adrenal was also performed.

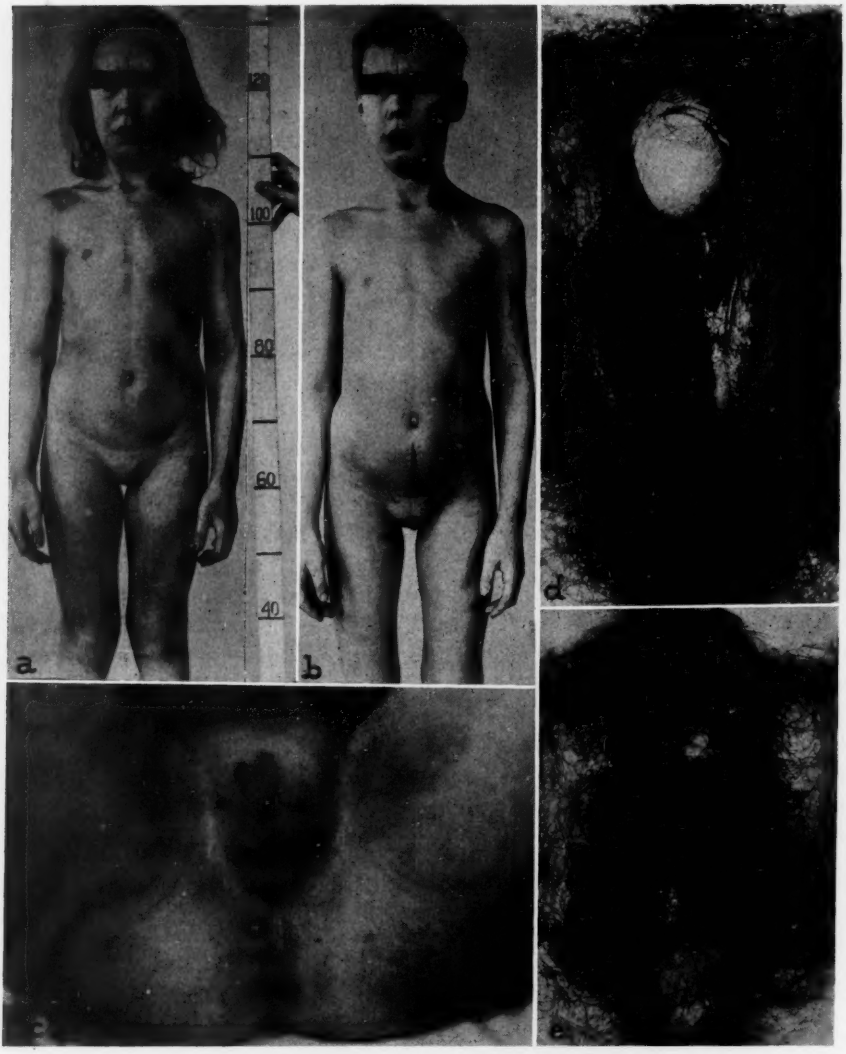

FIG. 6. *a*, *b*, *c*) Case 18, S. S., age 10. *d*) Case 19, R. F., age 17. *e*) Case 12, M. M., age 12.

The 3 cases present a variety of somatosexual aberrations. In Case 18, the patient was basically a male and erroneously brought up as a female until laparotomy (Dr. Cahill) revealed abdominal testes and absence of uterus or ovaries. In Case 19, photo *d* shows the "vulva" of a boy brought up as a girl until graduation from high school, Because "she" did not menstruate and was "manly" in appearance and voice, doubt arose as to the true sex. Laparotomy (Dr. Cahill) revealed a uterus and tubes, but biopsy of the

Pathology: left adrenal—adrenal tissue with areas containing large reticular cells; right adrenal—adrenal tissue with moderate hyperplasia of reticular zone. Urinary chlorides: 658 mg. NaCl/100 cc. (1280 mg. total NaCl in specimen) or 403 mg. Cl/100 cc. (783 mg. total Cl in specimen). Urinary 17-ketosteroids, reported several months later, were 43.2 mg./24 hrs. *Conclusion:* A female erroneously brought up as a boy because of a large clitoris. The true sex was revealed by operation and biopsy of gonads. The cause of the progressive masculinization was bilateral cortical hyperfunction and hyperplasia. Attempts to halt the process were made by partial resections.

Case 7, J. L., 591160. The patient was admitted at the age of 4 years because of an enlarged clitoris noticed at birth. Examination revealed a feminine-looking child with an enlarged clitoris and small vaginal orifice. No testes could be palpated. Roentgenograms of the skeleton revealed marked acceleration of maturation. Aerograms showed no enlargement of the adrenals. Urinary 17-ketosteroids were 14.8 mg./24 hours. Exploratory laparotomy (Dr. Cahill) revealed a uterus, tubes and gonads which proved to be ovaries on biopsy. The clitoris was then amputated. She has continued to grow rapidly for her age and to show evidence of masculinization. Urinary 17-ketosteroids showed a steady rise—35.5, 38.0, and 39.0 mg./24 hrs. on successive occasions. Hirsutism also appeared. Aerograms repeated at a later date revealed an enlarged left adrenal gland. The patient is to return for further study (Fig. 2 e, f). *Conclusion*: A female whose sex was in doubt from birth owing to an enlarged clitoris. That the masculinization was being effected by a cortical hyperfunction of the adrenal gland became evident as the patient was studied over a period of five years.

Case 8, M. H., 880166. The patient was admitted at the age of 7 years because of hirsutism, change of voice and an enlarged clitoris which had occurred within the previous fifteen months. Examination revealed a girl, large for her age, with axillary and profuse pubic hair, and large labia and clitoris. Urinary 17-ketosteroids were 173 mg./24 hrs. Roentgenograms of the skeleton showed accelerated maturation. Intravenous pyelography showed the right kidney pushed downwards by a soft tissue mass in the suprarenal region. Aerograms confirmed the presence of this mass. At operation (Dr. Robinson), a lemon-sized tumor of the right adrenal gland was found. Adrenalectomy was performed. *Pathology*: adenoma of adrenal cortex with preponderance of granular cells resembling those in the reticular zone. Postoperatively, there was a dramatic decrease in the urinary 17-ketosteroids to 2.6 mg./24 hrs. Three months later there was no evidence of neoplastic metastases (Fig. 5 a, b; Fig. 8 b). *Conclusion:* A young female who had undergone pronounced somatosexual alteration due to androgenic hormones secreted by cells of a cortical tumor. The remarkable drop in urinary 17-ketosteroids (from 173 mg./24 hrs. before operation to 2.6 mg./24 hrs. after operation) is of great interest.

Case 9, E. R. S., 867845. A 7-year-old girl was first admitted to the Urologic Service of Babies Hospital with a history of having developed pubic hair, a deep voice, a strong muscles over a period of fifteen months. Eight months previously, radiotherapy was given over each adrenal region, and about one year previously her physician performed a partial clitoridectomy, removing a piece about 1 inch long. Her blood pressure was 100/58, weight 31.0 Kg., and height 135 cm. Examination revealed a tall, healthy-look-

---

gonads showed that they were testes and not ovaries. In Case 12, photo *e* is that of a 12-year-old female with a corticosexual syndrome. Note similarity of "vulvae" in the last 2 cases. The cause in each, however, is different. In the one, a basic male shows retention of a Müllerian remnant (vagina); in the other a basic female shows Wolffian remnants which have been stimulated, resulting in a hypertrophied clitoris.

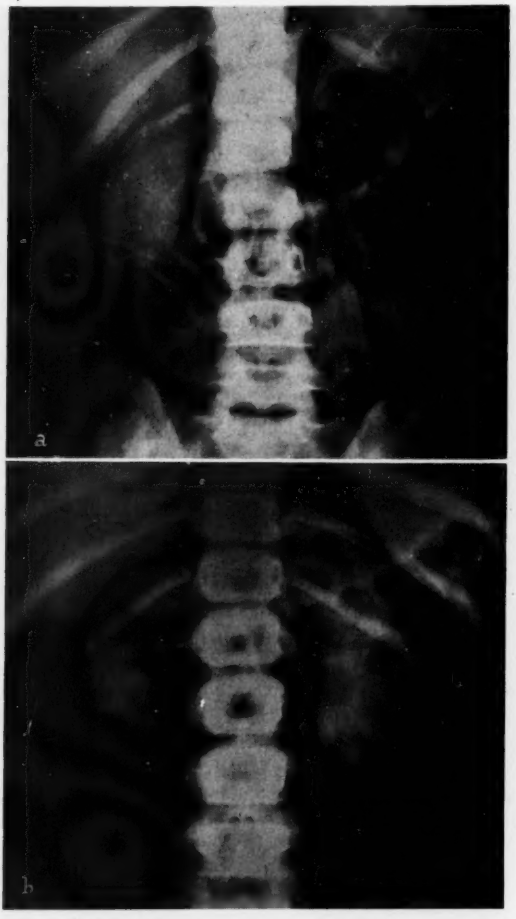

FIG. 7. Aerograms of adrenal glands. *a*) Case 12, M. M., age 12. Note enlargement of left adrenal. Patient had a corticosexual syndrome; urinary 17-ketosteroid output was 43 mg./24 hrs.; bone age was 20 years. *b*) Case 6, K. A., age 3½ years. Note bilateral enlargement of adrenal glands. Patient showed a pronounced corticosexual syndrome. A partial resection of each adrenal was done (Dr. Cahill).

ing, dark complexioned, black haired girl with a deep voice. The habitus was like that of a well developed 10-year-old boy. A moderate amount of curly black hair surrounded the stump of the enlarged clitoris. The rest of the examination did not reveal any other abnormalities. The breasts were not enlarged, and the vaginal orifice was small. There was no evidence of a prostate. Urinary 17-ketosteroids were 16.4 mg./24 hrs. Aerograms revealed an enlarged left adrenal gland (Fig. 8 a). At operation (Dr. Cahill), a tumor measuring 1.5×1 cm. was removed. The remainder of the adrenal gland was not excised. A uterus and ovaries were present. *Pathology:* adenoma of the adrenal cortex; ganglio-

FIG. 8. Aerograms of adrenal glands. *a*) Case 9, L. S., aged 7, with a corticosexual syndrome. Note enlargement of left adrenal gland. *b*) Case 8, M. H., aged 7, with a corticosexual syndrome due to a tumor of the right adrenal cortex.

neuroma. *Conclusion:* A female child whose femininity was being overpowered by a masculinizing influence arising from a hyperfunctioning cortical adenoma (corticosexual syndrome).

Case 10, C. A., 754001. A 7-year-old girl was admitted with a five months' history of progressive masculinization: acne, hirsutism, a gain of 15 pounds, deepening of the voice, increase in girth of the abdomen, and enlargement of the clitoris and labia majora. There was no history of menstruation. Examination revealed a muscular female, considerably older and larger than her stated age, and of masculine habitus (Fig. 5, c, d). The voice was deep, she had acne, and hair distribution was of the male type. The clitoris was considerably enlarged. Her blood pressure was 140/85. Urinary 17-ketosteroids were 76.8 mg./24 hrs. Roentgenograms of the skeleton showed accelerated maturation. Roentgenograms of the abdomen revealed a large mass with calcification in the right suprarenal region which had pushed the right kidney downwards. At operation (Dr. Cahill), a nonresectable tumor of the right adrenal gland was disclosed. *Pathology:* carcinoma of the

adrenal cortex. The patient expired seven days postoperatively. *Conclusion:* A female inundated by a masculinizing factor raising from a hyperfunctioning malignant growth of the right adrenal cortex. (corticosexual syndrome).

Case 11, C. A., 743115. This 7-year-old child was first admitted to Babies Hospital with a history of hypospadias which had been noted since birth, precocious puberty since the age of 4 years, and steadily increasing scoliosis. Five operations for the supposed hypospadias were performed elsewhere, the last being successful in establishing a penile urethra. Examination revealed a short, stocky, well developed "boy," profusely covered with coarse dark hair. The "penis" was greatly hypertrophied but no testes were demonstrable in the cleft scrotum nor was there a palpable prostate (Fig. 3, e, f). The blood pressure was 148/110, height 136 cm., and weight 38.0 Kg. On admission, the urinary 17-ketosteroids were 42.8 mg./24 hrs. *Roentgenograms* showed the following: hands—markedly accelerated maturation, equivalent to that at age of 25 years; pelvis—gynecoid; skull—accelerated development; spine—marked rotation and marked scoliosis in lower thoracic levels but no gross defect in vertebrae; sinuses—accelerated development (adult); all long bones—accelerated development of epiphyses. Aerograms showed presence of soft tissue mass in both suprarenal regions, possibly enlarged adrenals. Exploratory celiotomy (Dr. Cahill) revealed a uterus, tubes and gonads which, on biopsy, proved to be ovaries. In the right broad ligament, eight adrenal cortical rests were found ("Marchand bodies") (17). Subsequently, a partial right adrenocorticectomy was done. *Pathology:* hyperplasia of the cortex of the right adrenal gland. Six months later, the urinary 17-ketosteroids were 62.4 mg./24 hrs. An aerogram showed an enlarged left adrenal. *Roentgenograms* showed: chest—thoracic cage asymmetrical due to pronounced scoliosis of thoracic spine, right lung elongated and narrow, left lung broad, cardiac shadow widened and distorted, lung fields clear; spine—scoliosis of lumbar spine with convexity to the left. A partial left adrenocorticectomy was performed two years after the first operation because the left adrenal had become enlarged, and the patient's endocrine disturbance had not improved. *Pathology:* hyperplasia of the cortex of the left adrenal gland (biopsy). *Conclusion:* a basic female erroneously brought up as a boy because of a large clitoris. The underlying condition, a corticosexual syndrome emanating from hyperfunctioning right, left and aberrant adrenals, was not recognized until it was too late. The somatosexual mould was so altered that it became typically male, as did the physical, emotional and psychologic make-up. Complete hysterectomy and oophorectomy (Dr. Cahill) were therefore performed, resulting in a "neutral" but, at the same time, masculinized individual.

Case 12, M. M., 613899. The patient was admitted at the age of 12 years with a history of an enlarged clitoris, first noted at the age of 6 years. Doubt as to her true sex arose. During the previous few months, axillary, pubic and facial hair began to grow and her voice became coarse. The child was well developed and muscular. The clitoris was 2 inches long. The urethral orifice and vagina were in normal position (Fig. 6 e). Roentgenograms of the forearm showed complete fusion of the epiphyses, corresponding roughly to a bone age of about 20 years. Aerograms showed an indefinite enlargement of the left adrenal gland; the right appeared normal (Fig. 7a). Urinary 17-ketosteroids were 43 mg./24 hrs. Laparotomy (Dr. Cahill) revealed a uterus, tubes and ovaries; the clitoris was amputated. The patient was readmitted one year later and partial excision of the left adrenal was done (Dr. Cahill). *Pathology:* hyperplasia of the reticular zone type of cell of the adrenal cortex. *Conclusion:* A female whose femininity was being obscured by a a masculinizing process due to a corticosexual syndrome emanating from a hyperfunctioning hyperplastic left adrenal cortex.

B. In genetic males

There were 3 patients in this group, aged 2, 4½ and 6 years. All showed an acceleration toward puberty with an enlarged penis, adult scrotum, male pubic hairline, and advanced bone age. The urinary 17-ketosteroids were increased, particularly in the 6-year-old boy with an adenoma, whose urine assay showed 189 mg./24 hrs. All gave radiographic evidence of unilateral adrenal gland enlargement. Two of the patients were operated upon, one showing hyperplasia and the other tumor. The microscopic sections revealed a preponderance of granular cells, resembling the reticular zone.

Case 13, A. R. L., 701645. The patient was admitted at the age of 2 years because of enlargement of the penis and growth of pubic hair of two or three months' duration. Examination revealed a large child with a husky voice, large penis and small amount of pubic hair. Both testes were in the scrotum (Fig. 4a). Roentgenograms of the hands and knees showed accelerated development equal to a boy of 7 or 8 years of age. Aerograms showed enlargement of the right adrenal gland. Urinary 17-ketosteroids were 16.2 mg./24 hrs. Partial right adrenalectomy (Dr. Cahill) was performed. *Pathology:* hyperplasia of the granular cells, characteristic of the reticular zone of the adrenal cortex. The genitals showed regression when the patient was re-examined one year later. *Conclusion:* A "child Hercules," whose status was initiated by a hyperfunctioning hyperplastic right adrenal cortex (corticosexual syndrome).

Case 14, R. H., 654029—The patient was admitted at the age of 4½ years because of enlargement of the external genitalia, growth of suprapubic hair and large stature. The voice had become deeper. All the foregoing had developed within a period of three years. Examination revealed a boy taller than his stated age, with extensive suprapubic hair, large scrotum, small testes, and large penis (Fig. 4 b). Hair was present on arms and legs and in axillae. Urinary 17-ketosteroids were 18.8 mg./24 hrs. Roentgenograms showed a normal skull. Bilateral aerograms revealed no definite enlargement of either adrenal gland, but there was some suggestion that the left adrenal might be broader than usual. The boy had hypertension; his blood pressure was 160/115 and at a later date, 145/100. Muscle development was pronounced, giving him the appearance of an "infant Hercules." The penis was 7 cm. long and the prostate was palpable. Subsequent readmission revealed an elevation of urinary 17-ketosteroids to 29.6 mg./24 hrs. Six months after his first admission the blood pressure was 130/70. Urinary estrogens were 60 r.u./24 hrs. and gonadotropin, 5 m.u./24 hrs. The following laboratory tests were done: urinary sodium chloride—447 mg./100 cc. urine with a total of 483 mg. in the 108 cc. specimen; glucose tolerance test (glucose ingested, 55.6 Gm.)—control 84 mg., 30 min. 119 mg., 1 hr. 130 mg., 2 hrs. 107 mg., and 3 hrs. 97 mg. of glucose per 100 cc of blood; Wilder test—272.2 mg. per cent chloride in urine; psychometric test—IQ 126. The administration of stilbestrol was started and the genital disturbance abated. *Conclusion:* A male child with a somatosexual accentuation along androgenic lines. This was probably due to an overactivity emanating from the left adrenal cortex, but since the presence of a neoplasm or hyperplasia could not be demonstrated clearly by radiographs, operation was withheld. Estrogens in this case seem to have held the disturbance in abeyance. The hypertension subsided but the cause was not clear. It is possible that a corticometabolic factor was associated with the androgenic overactivity.

Case 15, F. H., 884922. This patient was admitted at the age of 6 years because of an enlarged penis and development of pubic hair of one year's duration (Fig. 4 c). Erections had occurred, the voice had become deep, and facial acne had appeared. The weight was 68 pounds, height 4 feet 6 inches, and the blood pressure 112/78. Urinary 17-ketosteroids were 189.0 mg./24 hrs. Roentgenograms showed a normal skull; and the elbows, knees and long bones showed a bone age of 14 years and 9 months. Aerograms revealed an enlarged left adrenal gland, suggestive of tumor. At operation (Dr. Cahill), an encapsulated tumor, weighing 147 Gm., was removed. *Pathology:* granular cell adenoma of the adrenal cortex, characteristic of that seen in the "adrenogenital" syndrome. Urinary 17-ketosteroids one year after operation were 5.0 mg./24 hrs; and one and one-half years after operation were 6.5 mg./24 hrs. *Conclusion:* a young boy with an androgenic drive originating from a tumor of the left adrenal cortex. The urinary 17-ketosteroids were remarkably increased, and dropped to normal after operation. This is a typical example of a somatosexual aberration developing in an otherwise normal child because of a functioning cortical tumor. The child stood the operation well and made an uneventful recovery.

GROUP III

Male pseudohermaphrodism

Four patients were not included in the foregoing classification because they presented no clinical, laboratory or radiographic evidence of cortical participation. However, a cortical estrogenic hyperfunction may have been temporarily present some time prior to the discovery of the somatosexual aberration. In 2, aged 3 and 6 years, the sex was in doubt. Two patients, aged 10 and 17 years were males brought up as females. The skeletal development of the group was normal for the chronologic age. The urinary 17-ketosteroids were normal in the cases in which the test was performed (Case 16, age 3, 1.6 mg./24 hrs.; Case 17, age 6, 1.2 mg./24 hrs). Radiographs of the adrenal glands showed no change from the normal. Since birth, each had what was identified as a large clitoris and cleft scrotum. The mother of the 10-year-old child began to doubt the child's alleged female status when she discovered a swelling in what was looked upon as the left labium. Exploratory laparotomy revealed no evidence of a uterus, tubes or ovaries. A vas deferens was present on each side and extended to undescended testes. The 17-year-old patient had been graduated from high school where "she" was looked upon as a "tomboy" because of athletic build and hairy skin. The phallus was large. When the voice became rough and menses did not start, medical advice was sought by parents and patient. Laparotomy demonstrated the presence of a uterus and tubes, but biopsy of the *gonads* revealed that they were *testes*. This individual was therefore a pseudohermaphrodite, genetically a male. The patient was originally admitted to the women's ward, but was transferred to a private room following the laparotomy. The long hair was cut, and a male name adopted. Later, the patient was readmitted, this time to the men's ward, for removal of a vestigial vagina, orchidopexy, and a plastic operation to

free the penis and construct a scrotum. Hysterectomy and salpingectomy were performed later. Histologic studies showed vaginal epithelium, and parts of a cervix, uterus, salpinx, prostate, epididymis, vas deferens, and seminal vesicles.

Case 16, J. K., 753473. This 3-year-old child was admitted because of hypospadias and cryptorchidism since birth. The mother had been told that the child was a pseudohermaphrodite and was advised to wait for several years before any operative procedure was instituted. Examination revealed a well developed white child, normal in height and weight for his chronologic age. He was not hirsute. He had a cleft scrotum and a small penis. The urethral opening was at the penoscrotal junction and no testes could be palpated (Fig. 2 b). Roentgenograms showed a normal genito-urinary tract and skull. Cystoscopy showed a normal bladder and trigone; the prostatic urethra had an enlarged orifice resembling the os of the uterine cervix and it admitted a catheter for a distance of 10 cm. The urinary 17-ketosteroids were 1.6 mg./24 hrs. Blood chemistry gave normal results. Exploratory celiotomy (Dr. Yeaw) was performed, revealing no gonad on the left side, but on the right side a testis could be palpated in the inguinal canal. There was no uterus. A sigmoidal tubular structure was present in the left side of the peritoneal cavity, and it extended down to the retrovesical space in the direction of the urethra. This structure was excised. *Pathology* (Dr. E. T. Engle): the duct proved to be an incomplete remnant of one of the embryonic genital ducts. On the basis of histologic characteristics, it was believed to be a Müllerian duct remnant, not differentiated. The patient was readmitted subsequently for plastic repair of the hypospadias and the undescended right testis. *Conclusion*: A pseudohermaphrodite, whose male status was determined by celiotomy, and in whom some estrogenic overactivity at one time, probably during intrauterine life, had retarded maleness and allowed Müllerian remnants to persist.

Case 17, E. P., 769849. The patient was admitted at the age of 6 years because of doubtful sex. The penis was small, with hypospadias, and the scrotum was bifid. A depression resembling a vagina was present. A laparotomy performed elsewhere had revealed no uterus or ovaries; bilateral masses were present in the inguinal regions which proved to be testes on biopsy. A colpogram showed a small vagina. The urinary 17-ketosteroids were 1.2 mg./24 hrs. Aerograms showed no enlargement of the adrenal glands. The false vagina was extirpated, and the hypospadias repaired (Dr. Yeaw); later, the chordee was corrected (Dr. Robinson). Three years later he was readmitted for further plastic repair of the penis (Dr. Barrett). Injections of gonadotropin, 1000 I.U. per week, were given to stimulate growth of the external genitalia. He is being followed in the clinic (Fig. 2 c, d). *Conclusion:* a basic male whose true sex was in doubt from birth. Laparotomy established the true sex. Operative procedures and hormonal therapy were instituted to accelerate masculine somatic appearance. It is possible that some estrogenic influence during gestation prevented adequate recession of Müllerian elements and retarded the Wolffian aspects.

Case 18, S. S., 860773. This 10-year-old child was raised as a girl, but doubt arose as to true sex, when the mother noted a swelling in the left labium. Examination revealed a normally developed alleged female. The labia majora were large, and a small depression was seen between them which resembled a vaginal opening. Two small masses could be felt in the upper part of both labia. The clitoris was considerably larger than normal for a female of that age. Bone maturation was normal. Exploratory laparotomy (Dr. Cahill) revealed the absence of a uterus, tubes and ovaries, but a definite vas deferens was seen on each side which went into the inguinal canals. It was decided to bring the testes down,

construct a scrotum, and raise the child as a boy. This was done, and biopsy of both gonads revealed undeveloped testes (Dr. E. T. Engle), characteristic of a 3 to 5-year-old boy (Fig. 6 a, b, c). *Conclusion:* A basic male erroneously brought up as a female because of a cleft scrotum simulating labia, and occult location of the gonads. The cause of the condition is not obvious. Some endocrine disturbance probably occurred during gestation which submerged the Wolffian elements.

Case 19, R. F., 831016. The patient was admitted at the age of 17 years, having been brought up as a female in spite of the fact that the external genitalia appeared abnormal since early infancy. Since the age of 12 years, the "clitoris" progressively enlarged, while the breasts failed to develop. Menses did not start. For two years prior to admission, body hair increased and was of male distribution. The patient was athletic, and though she had had several dates with boys, she never showed any emotional interest in them. Examination revealed a boyish "female" with mild acne, characteristic male hair distribution, and flat breasts. The "clitoris" measured 3 inches in length and $\frac{3}{4}$ of an inch in diameter. The urethra opened at the base, and no vagina was seen. In the left labium one could feel an oval, soft, movable, tender mass (Fig. 6 d). Roentgenograms showed a normal chest and genito-urinary tract. Aerograms revealed no adrenal enlargement. The urinary 17-ketosteroids were 6.4 mg./24 hrs. Cystoscopy revealed a normal bladder and trigone; the verumontanum was not seen nor could the prostate be identified. Rectal examination with a cystoscope in position confirmed the absence of a prostate. Exploratory laparotomy (Dr. Cahill) revealed a well developed uterus and normal right tube. The right gonad was small and proved to be a testis on biopsy (Dr. E. T. Engle). The left tube extended to the internal inguinal ring, and the adjacent gonad also proved to be a testis on biopsy. The adrenal glands were palpated and found to be normal. It was apparent that this patient was a male raised as a female. Therefore, it was decided to convert him into a more nearly normal male. He made an excellent psychologic adjustment and adopted a male name. He was readmitted to the men's ward for left orchidopexy and later for hysterectomy. *Pathology:* the findings were remarkable. They showed elements of both the Müllerian and Wolffian systems. The specimen consisted of a uterus, cervix, prostate, and seminal vesicles. *Conclusion:* A basic male erroneously brought up as a female because of absence of a characteristic scrotum containing gonads. When menses did not appear at puberty and the voice coarsened and the masculine build became accentuated, then doubt arose as to the patient's real sex, and "she" came to the hospital for investigation. This basic male possessed remnants of the upper Müllerian system (uterus, cervix), combined with incompletely formed and aberrantly placed Wolffian elements (intra-abdominal testes, epididymis, prostate). The cause is not apparent, but some endocrine influence (?disturbed estrogens) during intrauterine life may have been responsible.

GROUP IV

Cushing's syndrome

(Corticometabolic syndrome)

Case reports of infants and children with the stigmata of Cushing's disease are not rare. One of our patients (Case 20) may be placed under this heading. We wish to emphasize important differences between the adrenogenital (corticosexual) syndrome and Cushing's disease (corticometabolic). The outstanding finding in patients with a corticosexual syndrome is the sexual change due to excess androgens. They are emotionally

well-balanced and physically fit (virilism). Patients with Cushing's disease suffer predominantly from an adrenocortical metabolic disorder and are emotionally unbalanced and physically below par. The corticosexual patient is a good operative risk, the corticometabolic a poor one (18, 19). Many patients with Cushing's disease also show sexual changes, but the latter are the expression of a concomitant androgenic overactivity. It is this factor which confuses the picture and accounts for the case reports of adrenogenitalism with marked obesity. It is likely that many were primarily suffering from Cushing's syndrome with *incidental* sexual changes.

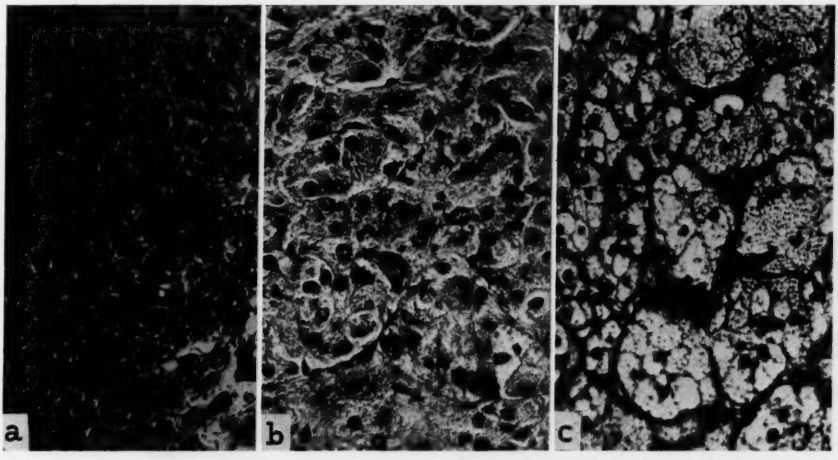

FIG. 9. *a & b*) Low and high power photomicrographs of adrenal tissue from a patient with a corticosexual syndrome. There is an increase in cells of the reticular zone. *c*) Photomicrograph in a case with Cushing's (corticometabolic) syndrome. Note large "foam cells" resembling those seen in the fascicular zone.

Our patient, aged 11, presented the characteristic "moon" face, "buffalo" neck, trunk obesity, striae, weakness, fatiguability and emotional instability, low basal metabolism, and low lymphocyte count of Cushing's disease. Radiographs revealed enlargement of the right adrenal gland. He died from a severe wound infection following exploration of the right adrenal. The microscopic picture of adrenal tissue removed from patients with Cushing's disease usually shows large masses of giant "foam cells" interspersed with groups of small granular (reticular) cells (Fig. 9 c).

Case 20, A. G., 572037. The patient was admitted at the age of 11 years with a history of hirsutism, flushed face, a weight gain of 30 pounds in a period of nine months, and severe headaches for five months. Examination revealed a typical moon face and buffalo neck. There were pads of fat over the abdomen and shoulders. At one time, a diagnosis of hypopituitarism had been made. There was hair on the face and in the armpits and pubic region. The face was plethoric and the cheeks very fat. The blood pressure was

FIG. 10. Case 20, A. G., age 11 years. A boy with Cushing's (corticometabolic) syndrome. Note moon face, fish mouth, narrow eye slits, trunk obesity, and hypogenitalism.

154/126, the height 138.5 cm., and the weight 51.5 Kg. The testes were well formed but the scrotum was small (Fig. 10 a, b, c). The skin on the legs became mottled when the patient was kept in an upright position. *Laboratory tests:* IQ 95; BMR—33.1 per cent; EKG showed no significant findings; serum:–carbon dioxide 76.6 vol. per cent or 32.4 mEq./L, chloride 576 mg. per cent or 98.6 mEq./L, inorganic phosphorus 3.3 mg. per cent or 1.9 mEq./L, protein 6.4 Gm. per cent, albumin 4.9 Gm. per cent or 13.4 mEq./L, globulin 1.5 Gm. per cent or 2.9 mEq./L, euglobulin 0.1 Gm. per cent, NPN 30 mg. per cent or 149.2 mEq./L, sodium 142.9 mEq./L, calcium 10.1 mg. per cent or 5.0 mEq./L, sugar 83 mg. per cent, cholesterol 230 mg. per cent, phosphatase 3.4 BU per cent; blood:–

hemoglobin 18.4 Gm. or 120 per cent, RBC 5,460,000, platelets and RBC appeared normal, WBC 12,650, P 74, L 16, M 7, E 3; urine:–straw, acid, sp. gr. 1.018, tr. alb., 0–4 wbc., and many rbc. *Roentgenograms:* skull:–pituitary fossa not enlarged, marked osteoporosis of dorsum sella with margins of floor of pituitary fossa demineralized posteriorly, suture lines closed, and no increase in digital markings; chest:–supracardiac shadow narrow, no suggestion of enlarged thymus, left lower lung hazy, with both lateral chest walls showing narrow zones of increased density suggestive of old pleural exudate; kidneys:–no calculi, but rounded soft-tissue shadow above and medial to upper pole of right kidney; adrenal:–enlarged on right. Intravenous pyelography revealed no evidence of pathology and no evidence of displacement of the right kidney or renal pelvis by the mass previously described; osteoporosis of the vertebral column was again conspicuous. Bilateral aerograms disclosed a small amount of air in the upper pole of the left kidney; the right kidney was not outlined; there was an area of increased density about 1½ inches in diameter overlying the upper pole of the right kidney and extending about 1 inch above it. A twenty-four hour film showed the area described still present; the left adrenal appeared normal. A right aerogram showed the same abnormality still present. The left adrenal was not enlarged. A forty-eight hour film showed the shadow still present on the right. Exploratory laparotomy (Dr. Cahill) was performed. The right adrenal was exposed but no tumor was noted; the left adrenal was explored by palpation. Several days following operation, wound infection developed, followed by peritonitis. The patient expired. *Conclusion:* A young male with pronounced somatic aberration due to a corticometabolic syndrome.

SUMMARY

Twenty cases with somatosexual aberrations have been presented.

A. There were 11 pseudohermaphrodites in whom true sex was determined by laparotomy and biopsy of the gonads:
   3 were females whose sex was in doubt.
   4 were females erroneously raised as males.
   2 were males whose sex was in doubt.
   2 were males erroneously raised as girls.
B. There were 8 patients with corticosexual disturbance:
   4 females with masculinization.
   4 males with precocious puberty.
C. There was 1 patient with Cushing's syndrome (corticometabolic).

We found that at birth and in early infancy the problem was mainly one of ascertaining the true sex and correcting the deviation. Several females were brought up as males and vice versa; in a number, the true sex was in doubt from birth. Awareness of error in some cases was not realized until puberty.

At birth and in early infancy, clinical, laboratory and radiologic evidence of cortical hyperfunction was usually not demonstrable. In some instances it became manifest later. Steps necessary to demonstrate cortical hyperfunction with or without neoplasia have been presented.

In childhood, the problem was mainly one of halting the tendency to

apparent sex reversal and removing the cause. The true sex had been ascertained at birth but, because of a profound heterosexual endocrine drive, a somatosexual aberration developed. Cortical hyperfunction was demonstrable in all cases.

CONCLUSIONS

1. The *true* hermaphrodite is probably a mutant and may be the result of a genetic error. The true hermaphrodite possesses gonads of both sexes (ovary and testis, or ovario-testis).

2. The *pseudo*hermaphrodite is genetically one sex, but somatically resembles the opposite sex (large clitoris, labiae and ovaries; or small penis, hypospadias, cleft scrotum, and abdominal or incompletely descended testes). Experimental and clinical evidence suggest that the condition may be the result of a prenatal endocrine disturbance. Somatosexual aberrations caused by excess androgens are usually of adrenocortical origin.

3. It is necessary to perform exploratory laparotomy with biopsy of the gonads in order to identify the true sex of a hermaphrodite.

4. The somatosexual pattern, extent and type of hirsutism, degree of bone maturation, assay of urinary 17-ketosteroids, and radiographs and aerograms of the adrenal glands are important in determining the role the adrenal cortex plays in the aberration.

5. Adrenocortical hyperfunction may be self-limited or indefinitely prolonged, and may or may not be associated with hyperplasia or neoplasia, benign or malignant, unilateral or bilateral.

6. The corticosexual syndrome (adrenogenitalism) results from a systemic inundation by androgens. There is acceleration of male puberty in boys, and masculinization of girls.

7. The corticometabolic syndrome (Cushing's syndrome) results from an excess of steroids that affect the "salt and water" balance and the metabolism of carbohydrates, proteins and fats. Variable degrees of androgenic overactivity may be associated.

REFERENCES

1. Greene, R. R.; Burrill, M. W., and Ivy, A. C.: Experimental intersexuality: the effect of estrogens on the antenatal sexual development of the rat, *Am. J. Anat.* **67**: 305–345, 1940.
2. Hamilton, J. B., and Wolfe, J. M.: The effect of male hormone substances upon birth and prenatal development in the rat, *Anat. Rec.* **70**: 433–440, 1935.
3. Turner, C. D.: The modification of sexual differentiation in genetic female mice by the prenatal administration of testosterone propionate, *J. Morphol.* **65**: 353–381, 1939.
4. Stoddard, F. J.: Hirsutism in pregnancy, *Am. J. Obst. & Gynec.* **49**: 417, 1945.
5. Saphir, W., and Parker, M. L.: Adrenal virilism, *J.A.M.A.* **107**: 1286, 1936.
6. Shepardson, H. C., and Shapiro, E.: The diabetes of bearded women (suprarenal

tumor, diabetes, and hirsutism): a clinical correlation of the function of the suprarenal cortex of carbohydrate metabolism, *Endocrinology* **24:** 237, 1939.

7. Woolley, G. W., and Little, C. C.: The incidence of adrenal cortical carcinoma in gonadectomized female mice of the extreme dilution strain. III. Observations on adrenal glands and accessory sex organs in mice 13 to 24 months of age, *Cancer Research* **5:** 321, 1945.
8. Bissell, G. W., and Williams, R. H.: Hirsutism in females; clinical study of its etiology, course and treatment, *Ann. Int. Med.* **22:** 773. 1945.
9. Neff, F. C.; Tice, G.; Walker, G. A., and Ockerblad, N.: Adrenal tumor in a female infant, with hypertrichosis, hypertension, overdevelopment of external genitalia, obesity, but absence of breast enlargement, *J. Clin. Endocrinol.* **2:** 125, 1942.
10. Reilly, W. A.; Lisser, H., and Hinman, F.: Pseudosexual-precocity; adrenal cortical syndrome in pre-adolescent girls; report of successfully operated case, *Endocrinology* **24:** 91, 1939.
11. Johnson, H. T., and Nesbit, R. M.: 17-Ketosteroids in the diagnosis of adrenal tumors, *Surgery* **21:** 184, 1947.
12. Mason, H. L., and Kepler, E. J.: Isolation of steroids from urine of patients with adrenal cortical tumors and adrenal cortical hyperplasia; new 17-ketosteroids, androstane-3 (a), 11-diol-17-one, *J. Biol. Chem.* **161:** 235, 1945.
13. Salter, W. T.; Cahen, R. L., and Sappington, T. S.: Urinary 17-ketosteroids in metabolism. II. Partition of gonadal and adrenocortical hormonal derivatives of normal, endocrine and cancerous patients, *J. Clin. Endocrinol.* **6:** 52, 1946.
14. Cahill, G. F.: Air injections to demonstrate adrenals by X-ray, *J. Urol.* **34:** 238, 1935.
15. Carelli, H. H.: Sur le pneumopéritoine et sur une méthode personelle pour voir le rein sans pneumopéritoine, *Bull. et mém. Soc. méd. d'hôp. de Paris* **45:** 1409, 1921.
16. Walters, W., and Kepler, E. J.: Adrenal cortical tumors and their treatment; study of 7 operated cases, *Ann. Surg.* **107:** 881, 1938.
17. Greene, H. J., and Lapp, W. C.: Adrenal rest tumor of ovary, *Am. J. Obst. & Gynec.* **47:** 63, 1944.
18. Rogoff, J. M., and Stewart, G. N.: Studies on adrenal insufficiency in dogs, *Am. J. Physiol.* **78:** 683, 711, 1926; *ibid.* **79:** 508, 1927; *ibid.* **86:** 25, 1928.
19. Weinberg, T.; Contralateral adrenal atrophy associated with cortical adrenal neoplasms, *New York State J. Med.* **41:** 884, 1941.

A COLOR TEST FOR DEHYDROISOANDROSTERONE AND CLOSELY RELATED STEROIDS, OF USE IN THE DIAGNOSIS OF ADRENO-CORTICAL TUMORS

WILLARD M. ALLEN, M.D., SAMUEL J. HAYWARD AND ASDRUBAL PINTO, M.D.*

From the Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, Missouri

THERE has been as yet no simple method for measuring urinary steroids which offers much help in the differential diagnosis of the conditions producing the adrenogenital syndrome. The steroids containing a keto group at C_{17} are readily measured by the color produced by reaction with m-dinitro-benzene (1); those conjugated with glucuronic acid can be measured either gravimetrically (2) or by determining the amount of glucuronic acid present (3), and the steroids can be actually isolated and identified. None of these procedures is simple, although the m-dinitro-benzene test for 17-ketosteroids is comparatively quick. The greatest shortcoming, however, of these methods is their failure, despite much purification and study of the fractions, to show a uniform difference between the two types of the syndrome in which hirsutism is most marked and in which the urinary excretion of the steroids is markedly increased, namely, adrenal cortical tumor and female pseudohermaphrodism with associated adrenal hyperplasia.

We have had the privilege of studying in considerable detail, during the past eight years, the excretion of urinary steroids in 5 cases of adrenal cortical tumor and in 7 cases of female pseudohermaphrodism, the diagnosis being proved correct in each case by appropriate operation. The excretion of the steroids in each of these cases has been significantly greater than normal, but there has been no uniform difference in the amount or kind of steroids which could be detected through determination of the 17-ketosteroids by the Zimmermann test, the steroid glucuronidates by the method of Allen and Viergiver (3) for sodium pregnanediol glucuronidate, or even by separation of the ketosteroids into alpha and beta types by precipitation with digitonin (4), (5).

This failure to detect any regular difference between these two types of

Received for publication June 1, 1949.

* Sharp and Dohme Fellow in Obstetrics and Gynecology.

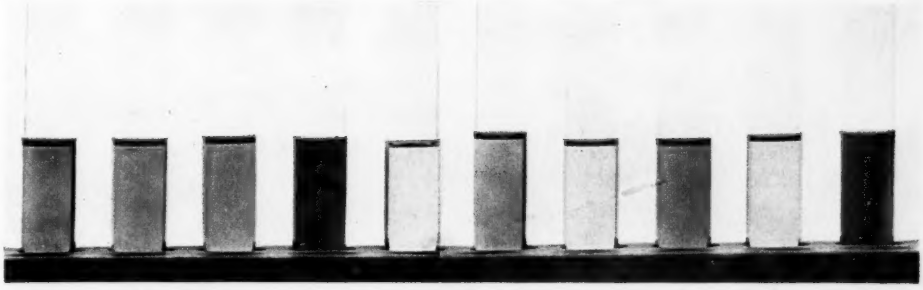

PLATE 1

Colors produced by heating urinary extracts of proven cases of adrenocortical tumor with 2 cc. of sulfuric acid-alcohol reagent for twelve minutes at 55°C. followed by the addition of 3 cc. of ethyl alcohol.

Case	Fraction of 24 hr. specimen	
1. M.O.	1/10,000	Before operation
2. M.O.	1/250	After removal of tumor
3. R.B.	1/5000	Before operation
4. R.B.	1/250	After removal of tumor
5. Reagent	Blank	
6. Y.N.	1/170	Before operation
7. Y.N.	1/100	After removal of tumor
8. D.Z.	1/200	Before operation
9. D.Z.	1/50	After removal of tumor
10. E.B.	1/83	Before operation

The color shown in 3 is virtually identical with that produced by .05 mg. of pure dehydroisoandrosterone. The brown color shown in 2 is similar to that produced by urinary extracts of cases of female pseudohermaphrodism with markedly elevated 17-ketosteroids, and of normal men and women.

The colors shown in 4 and 10 are those seen when small but detectable amounts of dehydroisoandrosterone are present. Later specimens of R.B. showed no detectable dehydroisoandrosterone.

the adrenogenital syndrome led us to explore a color reaction for dehydroisoandrosterone which was originally observed seven years ago while studying the types of color developed by various steroids, using a simple modification of the Kober test for estrogens. It was observed then that both androsterone and dehydroisoandrosterone gave a yellow color with concentrated sulfuric acid, but on dilution with ethyl alcohol the colors differed sharply; the color from dehydroisoandrosterone disappeared almost immediately on addition of the ethyl alcohol and was replaced in a few seconds by a comparatively stable dark purple color. Application of this test to extracts of urine from these patients with the adrenogenital syndrome showed that the purple color was produced in 4 of the 5 cases of adrenal tumor, and in none of the 7 cases of female pseudohermaphrodism. Interestingly enough, the one patient with adrenal tumor showing marked hypertension failed to give a grossly positive reaction to the test and this also was the one patient with virtually no increase in ketosteroids, but with a significant increase in steroid glucuronidate.

METHOD

The test is carried out as follows:

The urine is extracted three times with one-third volume of normal butyl alcohol. The butyl alcohol is distilled off in vacuo (water pump) leaving a dry residue which is then dissolved in 95 per cent ethyl alcohol as a stock solution. A suitable aliquot is evaporated to dryness on the steam bath and dissolved with 2 cubic centimeters of a special alcohol-sulfuric acid reagent. The reagent used is the one we use for determining estrogen by a modification of the Kober test. It is made by adding one volume of 90 per cent ethyl alcohol to four volumes of concentrated sulfuric acid. This reagent is stable and is much more convenient to use than concentrated sulfuric acid or phenolsulfonic acid, as in the Kober test. A brown, or brownish-red color appears immediately. The solution is then heated for twelve minutes at 55 degrees Centigrade. Not much additional color develops, although in some of the extracts from patients with adrenal tumor a faint green shade was observed. After the period of heating, the solution is cooled for one minute in cold water, and then 3 cubic centimeters of 95 per cent ethyl alcohol is added. An intense purple, reddish-purple, bluish-purple or blue color develops almost immediately. This can then be read in a colorimeter, the maximum absorption being at 600 mμ.

An alternate procedure for extraction is equally simple. The urine is hydrolyzed in the usual manner by boiling for ten minutes after adding 15 cc. of concentrated hydrochloric acid for each 100 cc. of urine. The neutral steroid fraction is then obtained by extracting the urine with an immiscible solvent, such as ether (or benzene), followed by washing the ether with 1.0 N aqueous sodium hydroxide. The ether is evaporated to dryness and the residue dissolved in 95 per cent ethyl alcohol as a stock solution. Any method for arriving at the neutral ketosteroid fraction is satisfactory. The test is carried out as previously described.

The amount of urine which needs to be extracted is small, since the impurities present make it impossible to carry out the test on more than 1/200 to 1/500 of a 24-hour specimen from an adult, when there is so little purification. This means, of course, that a good qualitative result can be obtained in the extract of no more than 5 cubic centimeters

of urine. Actually much less is needed, since in some of the tumors (described later) only 1/5000 of a 24-hour specimen was needed. Also it is not necessary to extract the urine more than once with butyl alcohol for good qualitative results. The conjugated steroid is much more soluble in butyl alcohol than in urine, so that a high percentage of the material is obtained by only one extraction.

RESULTS

The spectral transmission curves obtained from the butyl alcohol extracts of the urines from 2 patients with adrenal tumor (R. B. and D. Z)

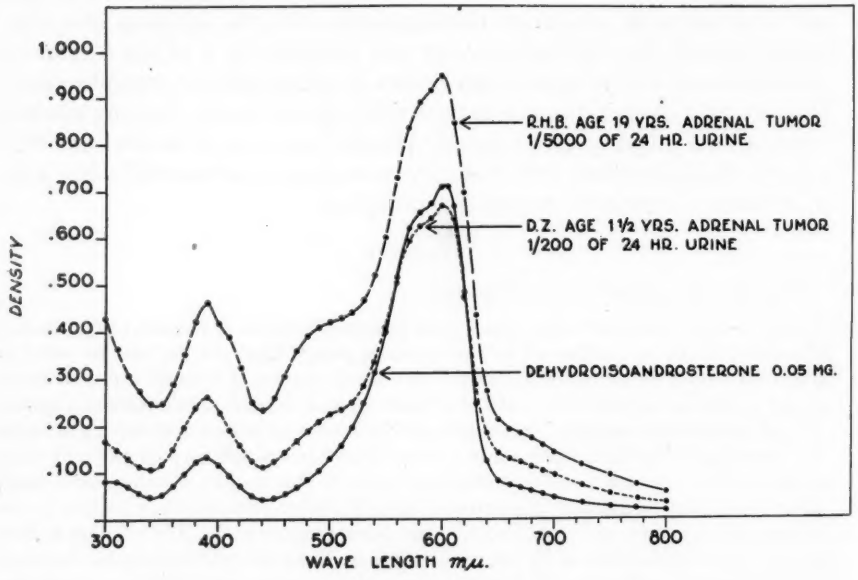

FIGURE 1

using the above described test, are shown for comparison with the curve obtained from pure dehydroisoandrosterone in Figure 1. These curves show the same point of maximum absorption at 600 mμ and minimal absorption at the same points. There is, however, a slight bulge over the region of 440 to 500 mμ in the curves obtained from the urinary extracts. The color produced by these two urinary extracts was virtually identical, to the eye, with that produced by dehydroisoandrosterone; and the transmission curves can be virtually superimposed on the curve obtained from pure dehydroisoandrosterone. It seems very probable, therefore, that the main compound giving the purple color in the urinary extracts of these 2 cases of adrenal tumor is dehydroisoandrosterone.

The color produced by the urinary extract of another patient (M.O.) was

somewhat different, being a deep blue rather than purple. The transmission curve also is considerably different (Fig. 2). There are two maxima, one at 605 mμ and another at 660 mμ. It seemed probable that this difference may have been produced by the presence of more than one reactive compound. This was proved to be the case by filter-paper chromatography. When a small amount of butyl alcohol extract was subjected to separation using a butyl alcohol-water system, two different substances were obtained,

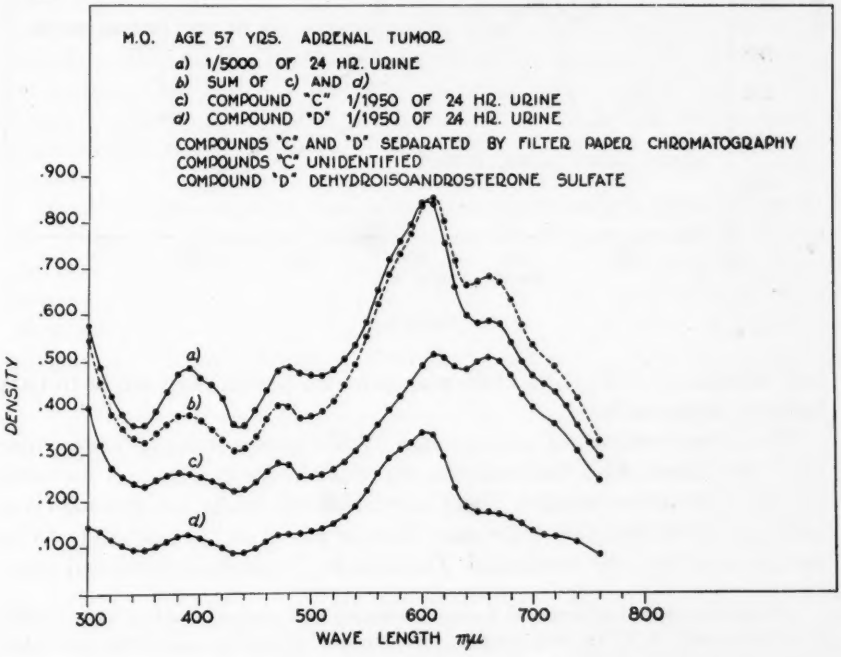

FIGURE 2

one which gave the characteristic purple color and absorption curve of dehydroisoandrosterone, and another which gave a dark blue color and showed maxima of about equal intensity at 610 mμ and 665 mμ. The first compound, which gives the color characteristic of dehydroisoandrosterone, is in all probability dehydroisoandrosterone sulfate. This compound (C) was soluble in water but, after hydrolysis, the chromogenic portion was readily extracted from the aqueous phase with ethyl ether. The aqueous fraction contained SO_4^{--} since on the addition of Ba^{++} an insoluble precipitate of $BaSO_4$ was obtained. The second compound (D) with the absorp-

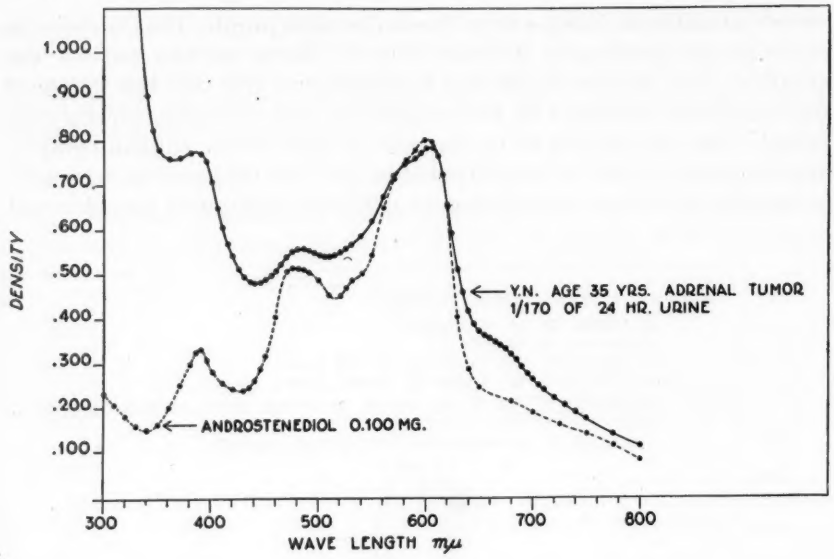

FIGURE 3

tion maxima at 610 mμ and 665 mμ, seems on preliminary study to be a hydroxy pregnenolone.[1]

The urinary extract of another case (Y.N.) gave a more lavender color than the others. This transmission curve is shown in Figure 3 for comparison with androstenediol. There is considerable similarity, although it is quite probable that this color may likewise prove on further study to be due to more than one compound. This case is of considerable clinical inter-

[1] Since this paper was prepared a very interesting and unusual case has been studied. A female infant (A.N.), aged 2 months, was admitted to the St. Louis Children's Hospital with the clinical picture of adrenal insufficiency but showed slight enlargement of the clitoris. The urine of this child gave a strongly positive "blue color test." The transmission curve obtained was virtually identical with that given by compound D. Subjection of this extract to filter paper chromatography gave only a trace of dehydroisoandrosterone at the proper place on the filter paper. The child was subjected to exploratory laparotomy even though her general condition was poor because of the strong probability that the disturbed metabolic state was due to an adrenal tumor. Both adrenal glands were markedly enlarged, being visibly larger than the kidney. The child succumbed several hours after operation and at autopsy both adrenals were as noted at operation but no definite nodular tumor was encountered. This case seems to be one, therefore, of very marked bilateral adrenal hyperplasia but with no congenital anomalies of the introitus such as are seen in female pseudohermaphrodism. It is of special interest because of the degree of hyperplasia, the adrenal insufficiency and the presence of large amounts of the unidentified steroid.

est also in that two specimens obtained several months following the removal of the tumor gave no purple, blue or lavender color and the transmission curves showed no maximum at 600 mμ, yet the patient died of metastases. Apparently the primary tumor was androgenic whereas the metastases were not.

The extract of the urine from 1 patient with a proven adrenocortical tumor (E.B.) failed to give a grossly positive result with the color test. This is shown in Figure 4, for comparison with a similar curve obtained from the urine extract of a patient with Cushing's syndrome (without adrenal tumor). These 2 patients had the moon face, obesity of the trunk, purple striae, and hypertension supposedly characteristic of Cushing's syndrome. One had a tumor, the other no tumor, yet clinically they appeared identical, and the urinary steroids as reflected in the spectral transmission curve of the color developed by the above test were very similar.

Finally, 6 patients with female pseudohermaphrodism showing the classic picture (*i.e.*, congenital adrenogenital syndrome) have been studied. Each of these patients also showed marked elevation of the urinary 17-ketosteroids and the steroid glucuronidates. The transmission curves obtained from the steroid fractions of extracts of the urine of 2 of these patients are shown in Figure 5. None of the cases of pseudohermaphrodism

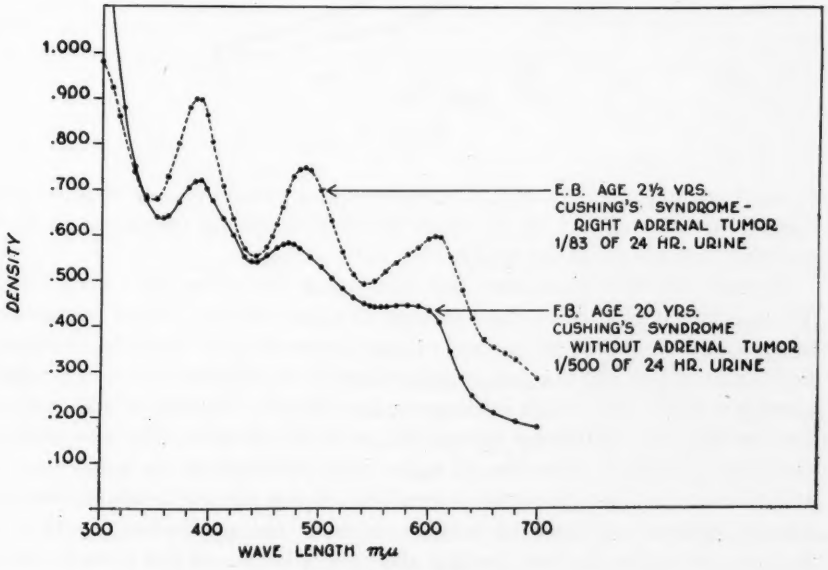

FIGURE 4

gave a blue or purple color, even though the amount of ketosteroid in the urine was approximately eight to ten times the normal amount. Also extracts of the urine of 3 pregnant women, 2 normal men, 1 man with acromegaly, and 6 women with pseudo-Cushing's syndrome gave negative results with the color test. Four of the 5 cases of adrenal tumor had, by the above described method, virtually no measurable dehydroisoandrosterone in the urine following removal of the tumor. Three of these also were found

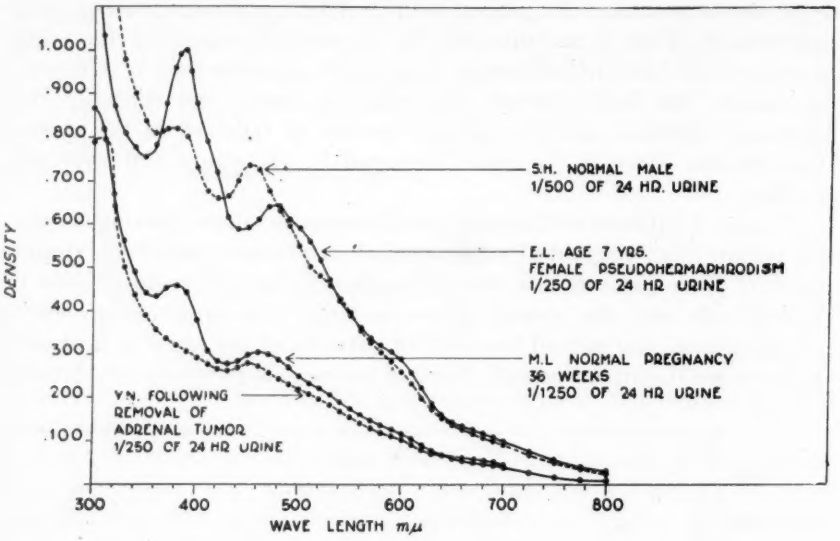

FIGURE 5

to have normal amounts of 17-ketosteroid and steroid glucuronidate after operation. One patient (E.B.) died one day following operation so that no urine was available for study.

Several different procedures for developing the color have been tried. The method described is the outcome of these studies. Minor variations in time and temperature are not of much consequence. Somewhat longer heating increases the amount of color slightly. A shorter period of heating gives less color. Too much heating, as for example, heating at 100 degrees for five minutes, markedly reduces the amount of color. The preliminary period of heating is, however, of major importance since virtually none of the characteristic purple color is produced if the alcohol is added directly to the sulfuric acid-alcohol reagent without the preliminary period of heating. Likewise, further heating after the addition of the 3 cubic centimeters of alcohol is not beneficial. The color is sufficiently stable to make

the readings easy. Even after twenty-four hours there is no more than 10 per cent loss of color. However, if the final solution of 5 cubic centimeters is diluted further with 5 cubic centimeters of alcohol the color rapidly fades.

The amount of dehydroisoandrosterone necessary to produce the proper amount of color for measurement by the Beckman spectrophotometer varies between 4 and 100 micrograms (in 5 cubic centimeters of solution; 2 cubic centimeters of the alcohol-sulfuric acid reagent plus 3 cubic centimeters of alcohol). Proportionality is good between 20 and 100 micrograms. Below 20 micrograms the amount of color produced is relatively somewhat less. The values given in Table 1 were obtained for the most part from

TABLE 1. CONSTANCY OF THE RELATIONSHIP BETWEEN DENSITY AND AMOUNT OF DEHYDROISOANDROSTERONE

Amount (micrograms)	Number of observations	K_1*	K_2**
100	8	81.3	152.2
80	5	87.0	168.1
50	6	83.2	161.3
40	4	91.3	178.6
25	6	83.9	160.2
20	2	85.7	168.7
Average, 20–100 micrograms		85.4	164.8
10	6	104.1	207.0
5	3	113.6	263.2

$$* K_1 = \frac{\text{Amount in micrograms}}{\text{DA at 600 m}\mu}$$

$$** K_2 = \frac{\text{Amount in micrograms}}{\left[\text{CDA}_{600} = \text{DA}_{600} - \frac{\text{DA}_{560} + \text{DA}_{640}}{2}\right]}$$

studies in which the constant temperature bath was only a vessel filled with water at 55 degrees Centigrade, obtained from the hot water tap and kept at approximately 55 degrees Centigrade for twelve minutes by adding either hot or cold water after placing the vessel on a warm steam bath. This was done intentionally to make the test as simple as possible. Ideally a standard of dehydroisoandrosterone (or the acetate) should be used for comparison with each unknown to secure better accuracy.

The actual calculation of the amount of dehydroisoandrosterone present in the sample from the absorption at 600 mμ is simple when the color pro-

duced is pure: when the color is contaminated, a correction is necessary. The usual formula for correction is not suitable because the contaminating color is variable. The density at 600 mμ, for example, does not bear a constant relation to the density at another wave length, say 700 mμ. A simple approximation, however, served to simplify the problem. The optical density is measured at the maximum of absorption (600 mμ) and at two other points (560 mμ and 640 mμ) equidistant from the maximum. Using these three densities, the following formula serves to give a fair approximation of the amount of the absorption at 600 mμ which can then be interpreted in terms of dehydroisoandrosterone:

$$CDA_{600} = OD_{600} - \frac{OD_{560} + OD_{640}}{2}$$

in which

CDA_{600} = calculated density at 600 mμ
OD_{600} = observed density at 600 mμ
OD_{560} = observed density at 560 mμ
OD_{640} = observed density at 640 mμ

In using this approximation it is, of course, necessary to use a standard curve in which the CDA_{600} for pure dehydroisoandrosterone is plotted against the amount, rather than the observed density at 600 mμ. This method simply "skims off" the peak between two adjoining points and assumes only that the contaminating color follows a straight line between 560 mμ and 640 mμ. (The theory regarding this simple approximation is presented elsewhere (6).)

This same method of calculation has been used to give an approximation in terms of dehydrosioandrosterone of the cases studied (Table 2). This table shows that the amount of steroids excreted and calculated as dehydroisoandrosterone was considerable in the patients with adrenal tumors, whereas in those with female pseudohermaphrodism the amount was small. There appears to be some overlapping between the groups, but if age is considered, it is seen at once that 2 of the 3 adults with adrenal tumors (R.B., M.O.) had more than 20 times as much dehydroisoandrosterone as the 2 adults with pseudohermaphrodism. The urine extract of the third adult (Y.N.) gave a strong blue color, but when the amount was calculated, only a little more dehydroisoandrosterone was found than was present in the urine of 1 of the cases of pseudohermaphrodism. However, comparison of the absorption curves leaves no doubt regarding the difference. The urine of the 2 children with adrenal tumors likewise had much more dehydroisoandrosterone than that of the 4 children with pseudohermaphrodism. The extract of the urine of 1 of the children with a tumor

TABLE 2. EXCRETION OF STEROIDS IN CASES OF ADRENAL TUMOR AND FEMALE PSEUDOHERMAPHRODISM

Case	Age (yrs.)	NaXG* (mg.)	Total** 17-keto-steroids (mg.)	Dehydro-isoandro-sterone† (mg.)	Color	Aliquot of 24-hr. urine used for color test
			Adrenal tumors (all in females)			
M.O.	57	234	120	161	blue	1/10,000
R.B.	19	300	392	363	purple	1/5,000
Y.N.	35		67	8	lavender	1/170
D.Z.	1½	14	52	54	purple	1/200
E.B.	2½	19	2	17	purplish-brown	1/83
			Adrenal hyperplasia (female pseudohermaphrodism)			
L.C.	33	130	70	7	brown	1/400
F.A.	23	127	77	5	brown	1/250
E.L.	7	66	14	2	brown	1/250
N.K.	6	70	12	0.4	brown	1/50
M.E.	5½	58	8	2	brown	1/167
G.T.	4	22	2.5	0.4	brown	1/134
J.C.	9 mo.	14	3	0	brown	1/125

* NaXG determined by method of Allen and Viergiver (3). Values calculated as sodium pregnanediol glucuronidate. X indicates the uncertain nature of the steroid conjugated with glucuronic acid.

** Total 17-ketosteroids determined by modification of the Zimmermann (1) method, without correction for color produced by impurities.

† Values for dehydroisoandrosterone obtained by "skimming the peak" at 600 mμ using K_2 (Table 1).

(E.B.) gave a negative result, due to contaminating yellow colors, but when subjected to analysis was shown to have a considerable amount of dehydroisoandrosterone. Actually this 2½-year-old child excreted more than the adult (F.B.) with Cushing's syndrome, without an adrenal tumor.

The specificity of this color reaction is naturally of interest (Figs. 6 and 7). A fair representation of the three great classes of steroids, the saturated compounds, the alpha-beta ketones and the compounds with a double bond in ring B have been tested. These results are given in Table 3. The densities at 480 mμ and 600 mμ are recorded, since these are the points where significant maxima may occur. The values given are the observed ones in some instances and in others calculated to indicate the relative densities from the same amount of material, namely, 100 micrograms in 5 cubic centimeters of solution. In each case a density of .100 or more was accompanied by a maximum at the indicated wave length.

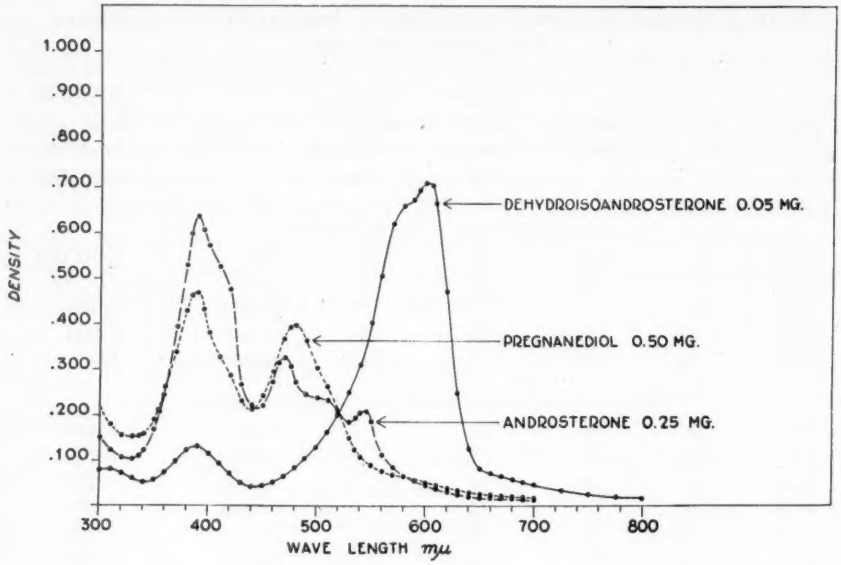

FIGURE 6

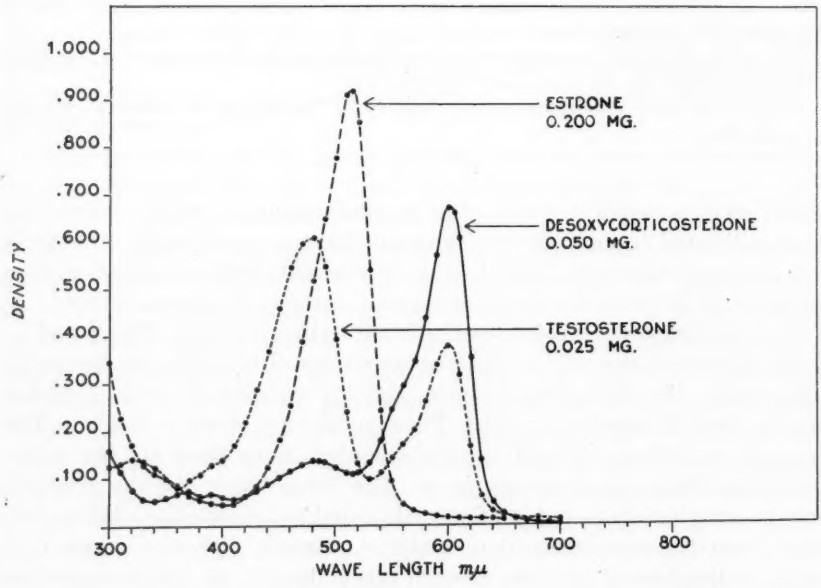

FIGURE 7

TABLE 3. DENSITY OF COLOR PRODUCED BY VARIOUS STEROIDS (CALCULATED FOR 100 MICROGRAMS EACH) AFTER TREATMENT WITH 2 CC. SULFURIC ACID-ALCOHOL REAGENT FOR TWELVE MINUTES AT 55° C. FOLLOWED BY 3 CC. OF 95 PER CENT ETHYL ALCOHOL*

Compound	D_{480}**	D_{600}**	CD_{600}†
3 Keto Δ4,5 compounds, alpha-beta ketones			
Testosterone	2.420	1.500	1.100
Desoxycorticosterone	.270	1.336	.995
Androstenedione	.652	.460	.319
Progesterone	.046	.033	.022
17-Hydroxy 11-dehydrocorticosterone	.110	.015	.006
3 Hydroxy Δ5,6 compounds			
Dehydroisoandrosterone	.169	1.230	.658
Androstenediol	.512	.800	.333
21-Acetoxy pregnenolone	.304	.470	.121
Pregnenolone	.188	.068	.000
17-Methyl androstenediol	.150	.022	−.004
Saturated compounds			
Pregnanediol	.079	.010	−.001
Pregnanolone	.023	.006	.000
Androstanediol	.086	.018	−.004
Androsterone	.108	.027	−.009

* Using square cuvettes 1 cm. deep in the Beckman spectrophotometer.

** D_{480}, D_{600} are observed densities at 480 mμ and 600 mμ.

† $CD_{600} = OD_{600} - \frac{OD_{560} + OD_{640}}{2}$. The magnitude of this calculated value serves as an index of the sharpness of a maximum at 600 mμ. The absence of a positive value indicates that the absorption curve is virtually a straight line between 560 mμ and 640 mμ.

Study of Table 3 shows immediately that none of the saturated compounds listed shows any maximum at 600 mμ. Although they do give some absorption, the use of the method of "skimming the peak" eliminates them completely as a cause for absorption at 600 mμ. This means that, for all practical purposes, the saturated compounds of the androstane and pregnane series do not contribute to the maximum observed at 600 mμ. Secondly, some of the unsaturated compounds of both series have maxima at 600 mμ. However, the alpha-beta ketones presumably do not occur in the urine so that the compounds being measured by this color test are probably those having a hydroxyl group at C_3 and a double bond in ring B, the chief one being dehydroisoandrosterone. There is one other point. The addition of a hydroxyl group in the short side chain of pregnenolone (21 hydroxy pregnenolone) or of progesterone (21 hydroxy progesterone,

i.e., desoxycorticosterone) renders the compounds chromogenic. This suggests that if a hydroxy pregnenolone were present in the extract it might be expected to give a purple or blue color.

The compounds with a double bond in ring B are also reactive and are naturally of much more significance in so far as the urinary steroids are concerned. Dehydroisoandrosterone gives a brilliant purple color with an absorption band between 420 mμ and 650 mμ, having a maximum at 600 mμ. Androstenediol shows a similar band, but there is an additional but smaller maximum at about 480 mμ. This secondary band can be obtained

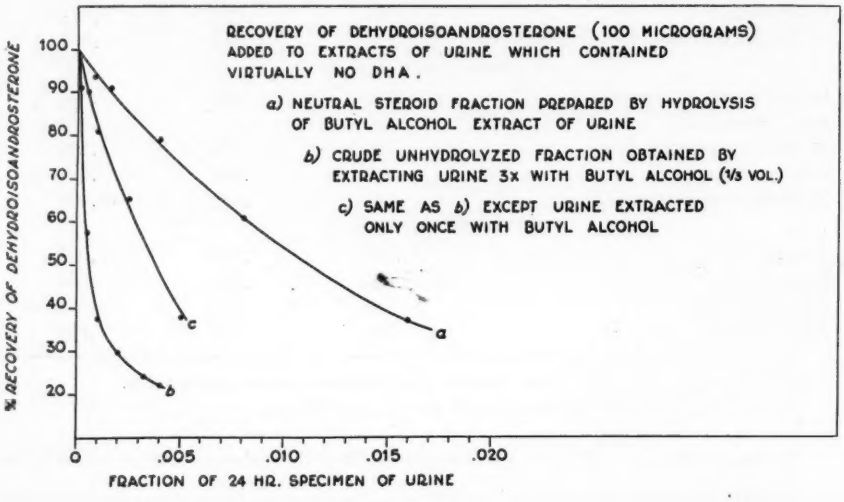

FIGURE 8

with dehydroisoandrosterone by modifying the conditions of the reaction. However, the method described was actually developed by trial and error so as to eliminate this secondary band, and thereby obtain as pure a color as possible with dehydroisoandrosterone. It is quite possible, therefore, that modification of the conditions of the reaction might eliminate this secondary absorption band obtained with androstenediol and give a color identical with that given by dehydroisoandrosterone. The replacement of the hydrogen atom at C_{17} in androstenediol by a CH_3 group prevents the appearance of an absorption band in the region of 600 mμ. Likewise, the replacement of an hydroxyl group at C_{17} by CH_3CO (pregnenolone) or by a long saturated side chain (cholesterol) or a long side chain containing a double bond (stigmasterol) abolishes the reactivity of the compound.

The values recorded in Table 2 for the excretion of dehydroisoandrosterone do not represent the actual amounts, because no attempt has been

made to correct for the inhibiting effect of contaminating substances on the development of the color. The presence of contaminating substances does prevent the development of the expected amount of color. This is shown graphically in Figure 8. These data were obtained by adding 100 micrograms of dehydroisoandrosterone to each of several dilutions of three different urinary extracts, each of which contained little or no dehydroisoandrosterone. Even with as little as 1/1000 of a 24-hour specimen obtained by extraction of the urine three times with butyl alcohol the recovery was only 37 per cent. With 1/5000, however, of this fraction the recovery was 92 per cent. This indicates that the values obtained from the extracts of the urine of some of the cases of adrenal tumor may be reasonably accurate. Further purification of the urinary extract by preparation of the neutral steroid fraction in the usual manner removes a much larger amount of the contaminating substances which interfere with the development of the color. Curve "a" shows the recoveries when such a fraction was used. Approximately 50 per cent recovery of 100 micrograms can be expected when 1/100 of a 24-hour specimen is used. Many more studies will be necessary, of course, before the percentage recovery under various conditions can be predicted.

DISCUSSION

There are obvious irregularities in the results recorded in Table 2 which only serve to emphasize the complex nature of the problem of steroid excretion. For example, 1 patient (M.O.) with an adrenal tumor excreted 234 milligrams of steroid glucuronidate, equivalent to approximately 135 milligrams of free steroid. This correlates rather well with the observed amount of 120 milligrams of 17-ketosteroids. Another patient with a tumor (R.B.) excreted 300 milligrams of steroid glucuronidate equivalent to approximately 175 milligrams of 17-ketosteroids, yet the total 17-ketosteroids measured 392 milligrams and the dehydroisoandrosterone, 363 milligrams. These two studies support the observations of others, however, that a high percentage of the total 17-ketosteroids in some cases of adrenal tumor may be present in the digitonin-precipitable (dehydroisoandrosterone) fraction. Also, since there is much more steroid in the second case than can be accounted for in the steroid glucuronidate fraction, it seems probable that the additional steroid is conjugated with sulfuric acid. The 7 cases of pseudohermaphrodism gave results which are in accord with the usual findings; the dehydroisoandrosterone is no more than 10 per cent of the total 17-ketosteroids. A further point of interest is that 1 of the children with an adrenal tumor (E.B.) gave a low value for 17-ketosteroids, a high value for dehydroisoandrosterone and a high value for steroid glucuronidate. It seems very probable that in this case the steroid which gives the adsorp-

tion at 600 mμ, and which is determined as dehydroisoandrosterone, is not a ketosteroid but rather a nonketonic compound some of which is conjugated with glucuronic acid.

The separation of the steroids into alpha and beta forms by precipitation with digitonin has not been very helpful in differentiating patients with adrenal tumor from those with adrenal hyperplasia. The earlier cases were studied by this method (4, 5). One patient with a tumor (R.B.) showed from 20 to 50 per cent of the steroids in the digitonin-precipitable form (beta); but 1 of the patients with pseudohermaphrodism (F.A.) also had 30 per cent of the ketosteroids in the digitonin-precipitable form. However, when the urines of the same 2 patients were compared, using the sulfuric acid color test, the difference between the two became apparent. The urine of the one with a tumor (R.B.) gave a strongly positive color for dehydroisoandrosterone, whereas the other gave a negative color. Also the amount of urinary dehydroisoandrosterone in the patient with a tumor was nearly as great as the amount of total 17-ketosteroids; whereas, in the patients with pseudohermaphrodism the urinary dehydroisoandrosterone was only 6.5 per cent of the total of the 17-ketosteroids.

Finally, if any one of the tests is to be used, it is apparent that the production of a purple or blue color with the special sulfuric acid reagent correlates better with the presence of an adrenal tumor than do any of the other tests. Four of the 5 cases gave a grossly positive result with the color test, whereas all of the cases of pseudohermaphrodism gave a negative result. To be sure, the urine of 1 of the children with a tumor (E.B.) gave a negative result; but when the spectral transmission curve was analyzed this patient, too, proved to have much more material giving absorption at 600 mμ than did any of the 3 children, or even the 2 adults with pseudohermaphrodism.

The preliminary studies regarding the specificity of the color indicate that the absorption band with a maximum at about 600 mμ produced by the urinary extracts is probably due to a small group of structurally closely related compounds. These compounds, having an hydroxyl group at C_3, a double bond in ring B, and either oxygen at C_{17} (dehydroisoandrosterone, androstenediol) or two oxygen atoms in a short side chain (hydroxy pregnenolone), seem the most likely urinary steroids responsible for the absorption maximum at 600 mμ.

A detailed study of the application of this color test for dehydroisoandrosterone, and closely related compounds giving similar colors to steroid fractions of the urine, has not as yet been made. Certain pertinent information, however, has been obtained. The compound (or compounds) responsible for the color, is not extractable from aqueous solutions with ether or benzene prior to hydrolysis, so that it is undoubtedly present in a conjugated, water-soluble form. After hydrolysis, however, the compound

is readily extracted with ether or benzene. The conjugated compound is quite soluble in butyl alcohol and appears to be completely extracted from the urine by three extractions with one-third volume of butyl alcohol.

While this study was in progress a very important paper by Patterson (7) appeared in *The Lancet*. The method reported in this paper is very similar to the one we have used. Patterson's method is as follows:

> A 25 cc. aliquot of urine is hydrolyzed and extracted with ether. The ether is washed with 10 per cent sodium hydroxide, aqueous sodium hydrosulfite and water. The ether is evaporated to dryness and the residue dissolved in 1.0 cc. of absolute ethanol. Two tenths of a cc. of this solution is used for determination of the 17-ketosteroids. Another aliquot of 0.2 cc. is used for the development of the blue color characteristic for dehydroisoandrosterone. This is evaporated to dryness, followed by the addition of 2.0 cc. of concentrated sulfuric acid. This solution is heated at 25 degrees Centigrade for twenty minutes, cooled, 1.0 cc. of water is added slowly, and the diluted solution heated for one minute on the steam bath. The blue color appears immediately.

Patterson has obtained positive results with the test in 3 cases of adrenal tumor, negative in 6 cases of prepuberal virilism, and negative in 8 cases of acquired virilism with 17-ketosteroids varying from 21 to 30 milligrams per day.

The color produced by dehydroisoandrosterone, using this modification of the Kober test (8), appears to have been first reported by Descheryl and Zilliken in 1943 (9). Their procedure for the development of the color is virtually identical with that used by Patterson. They state that androsterone, testosterone, androstenediol, estrone, estradiol and progesterone give negative results. It is of interest, however, that isoandrostanol-6-one-17 gives a blue color. This is a saturated compound which may be reactive because of the hydroxyl group attached to C_6. In the course of the treatment with the sulfuric acid, water may be removed and a double bond introduced in ring B.

Patterson's results, together with those reported in this paper, indicate that the color test has given grossly positive results in 8 cases of adrenal tumor (Patterson—3; this paper—4; Henry Turner (personal communication)—1) and negative results in 1 case (E.B.). The case in which the results were negative, however, shows much more dehydroisoandrosterone than normal. The results have also been found negative in 13 cases of prepuberal virilism (Patterson—6; this paper—7). These observations suggest, of course, that this very simple color test should be of considerable practical help in the diagnosis of adrenocortical tumors.

SUMMARY

A simple color test for urinary dehydroisoandrosterone and certain other compounds having a double bond in ring B is described. The test can be used on either butyl alcohol extracts or ether extracts (after hydrolysis) of the urine and is carried out by adding 2 cubic centimeters of a special

sulfuric acid reagent (four parts concentrated sulfuric acid to one part of 90 per cent ethyl alcohol) to the dry residue of a suitable aliquot of the urine (usually one two-hundredths or less of a 24-hour specimen) followed by heating at 55 degrees Centigrade for twelve minutes. The addition of 3 cubic centimeters of 95 per cent alcohol causes the prompt appearance of a purple or blue color with an absorption maximum at about 600 mμ. A purple color was given by such crude extracts of the urine of 4 out of 5 cases of proven adrenocortical tumor. A brown color was produced by the urinary extracts of 6 cases of female pseudohermaphrodism, all showing ketosteroid levels much greater than normal.

Acknowledgments

The authors are indebted to the following physicians for the privilege of studying many of these cases: Ephraim Shorr for Y.N., Lauren V. Ackerman for M.O., Paul Zentay for D.Z., Joseph C. Jaudon for G.T. and A.N., Jean Klingberg for J.C., F. P. McNally for N.K., S. D. Soule for L.C., and Willard C. Scrivner for F.A.

Heinbecker has included case E.B. in a paper dealing with Cushing's syndrome (his case No. v (10)). The other cases of adrenal tumor have not been previously reported. Cases L.C., F.A., N.K. and E.L., of female pseudohermaphrodism have been briefly considered elsewhere (11).

The authors are indebted to the Schering Corporation, Ciba Pharmaceutical Products, Inc., and the Glidden Company for the steroids used in developing the color test.

REFERENCES

1. Zimmermann, W.: Colorimetrische Bestimmung der Keimdrüsenhormone, *Ztschr. f. physiol. Chem.* **245:** 47, 1936.
2. Venning, E. H.: Further studies on estimation of small amounts of sodium pregnanediol glucuronidate in urine, *J. Biol. Chem.* **126:** 595, 1938.
3. Allen, W. M., and Viergiver, E.: A titrimetric method for the determination of sodium pregnanediol glucuronidate in the urine of pregnant women, *J. Biol. Chem.* **141:** 837, 1941.
4. Frame, E. G.: A micromethod for the separation of 17-ketosteroids into alpha and beta fractions, *Endocrinology* **34:** 175, 1944.
5. Langstroth, G. O.; Talbot, N. B., and Fineman, A.: Spectrochemical assay of androsterone and dihydroisoandrosterone in simple solutions, *J. Biol. Chem.* **130:** 585, 1939.
6. Allen, W. M.: A simple method for analyzing complicated absorption curves, of use in the colorimetric determination of urinary steroids, *J. Clin. Endocrinol.* **10:** 71, 1950.
7. Patterson, J.: Diagnosis of adrenal tumours; new chemical test, *Lancet* **2:** 580, 1947.
8. Kober, S.: Ein kolorimetrische Bestimmung des Brunsthormons (Menformon), *Biochem. Ztschr.* **239:** 209, 1931.
9. Dirscherl, W., and Zilliken, F.: Eine Farbreaktion des trans-dehydro Androsterone, *Naturwissenschaften* **31:** 349, 1943.
10. Heinbecker, P.: Pathogenesis of Cushing's syndrome, *Medicine* **23:** 225, 1944.
11. Herweg, J. C.; Ackerman, L. V., and Allen, W. M.: The excretion of neutral 17-ketosteroids in two cases of male pseudohermaphroditism, *J. Clin. Endocrinol.* **6:** 275, 1946.

A SIMPLE METHOD FOR ANALYZING COMPLICATED ABSORPTION CURVES, OF USE IN THE COLORIMETRIC DETERMINATION OF URINARY STEROIDS

WILLARD M. ALLEN, M.D.

From the Department of Obstetrics and Gynecology, Washington University School of Medicine, Saint Louis, Missouri

THERE are many color reactions which have been found useful in the quantitative determination of several of the steroids. The first of these, employing phenolsulfonic acid, was discovered by Kober in 1931 (1) and has been employed in various modifications for the determination of the urinary estrogens. The second, utilizing m-dinitrobenzene, was discovered by Zimmermann in 1936 and has become very useful for the determination of the urinary 17-ketosteroids (2). Both of these reactions give characteristic colors with specific absorption maxima when pure compounds are used. However, in urinary extracts there are chromogenic impurities which make a correction necessary unless the compounds being measured are present in considerable amount. These two color reactions, and modifications thereof, have actually almost completely replaced bio-assay as a method for studying the excretion of the steroids. Also they are in many instances more valuable than bio-assay since they make possible the study of relatively inactive steroids.

The need for accurate determination by colorimetric means has produced various methods for the elimination of the color due to impurities. The usual procedure employs a correction factor obtained by a separate study of the absorption spectra of the pure compound and the contaminating substances. This is possible when the methods of extracting the urine are such that the impurities are virtually always the same and hence give the same nonspecific color when treated with the reagent used for developing the specific color by the compound being determined.

The usual formula for this correction is (3)

$$DE_a = \frac{OD_b - K_I OD_a}{K_E - K_I}$$

Where DE_a = density due to estriol (using estriol as an example), at wave length $_a$, usually the point of maximum absorption of estriol.

OD_b = observed density at wave length $_b$, usually a point of maxi-

Received for publication June 1, 1949.

mum absorption of the impurities and minimum absorption of the estriol.

OD_a = observed density at wave length $_a$

$$K_I = \frac{\text{density of impurity at wave length } _b}{\text{density of impurity at wave length } _a}$$

$$K_E = \frac{\text{density of estriol at wave length } _b}{\text{density of estriol at wave length } _a}$$

Simple inspection of this formula shows that the accuracy is dependent on the constancy of the values for the constants in the equation. The major shortcoming of this formula is the inconstancy of the value for K_I. This may vary a great deal, even when dealing with the same fraction of urine. For example, in one study of the excretion of estrogens the K_I varied from 1.07 to 2.53 in the same fraction from the same patient on different days; in another fraction, from 1.11 to 2.27; and in a third, from 1.15 to 1.76. In another study in which the excretion of dehydroisoandrosterone was being determined, the K_I varied from 1.96 to 7.72. This variability is so great that the use of this formula for determining the color produced by the impurities may give large errors when the amount of the substance being measured is small.

The need for a better formula is obvious. An ideal formula for correction should be one in which the optical density due to the impurity can be subtracted accurately and simply from the total density with as few limitations as possible. In the above formula the assumption is made that the optical density at one wave length bears a constant relationship to the optical density at another, an unwarranted assumption. However, another assumption can be made which makes reliable calculation possible. Inspection of a large number of spectral absorption curves obtained by heating suitable urinary extracts with a special sulfuric acid reagent (one part 90 per cent ethyl alcohol to four parts concentrated sulfuric acid) shows that those extracts which contain no estrogen give an absorption curve which very closely approaches a straight line in the critical range between 380 mμ and 520 mμ. (The specific absorption maximum with estriol is at 450 mμ when this modification of the Kober test is used. The color is developed by heating the dried residue of the urinary extract for ten minutes at 100 degrees with 2 cubic centimeters of the reagent, followed by dilution with the reagent to a convenient volume, say 5 or 10 cc.) Since the lines are straight, an entirely different method of correction becomes feasible. This new method has now been in use for several years in this laboratory.

More recently studies have been made on the use of this sulfuric acid reagent for the determination of dehydroisoandrosterone (4). By a simple

modification of the procedure, the Kober test becomes applicable to the quantitative determination of dehydroisoandrosterone and is clinically useful in the differential diagnosis of adrenal tumor and adrenal hyperplasia. In these studies also there is need for a formula for color correction. The curves showing no dehydroisoandrosterone likewise seemed closely to approximate a straight line in the zone between 520 mμ and 700 mμ. (The absorption maximum is at 600 mμ.) Consequently the same formula

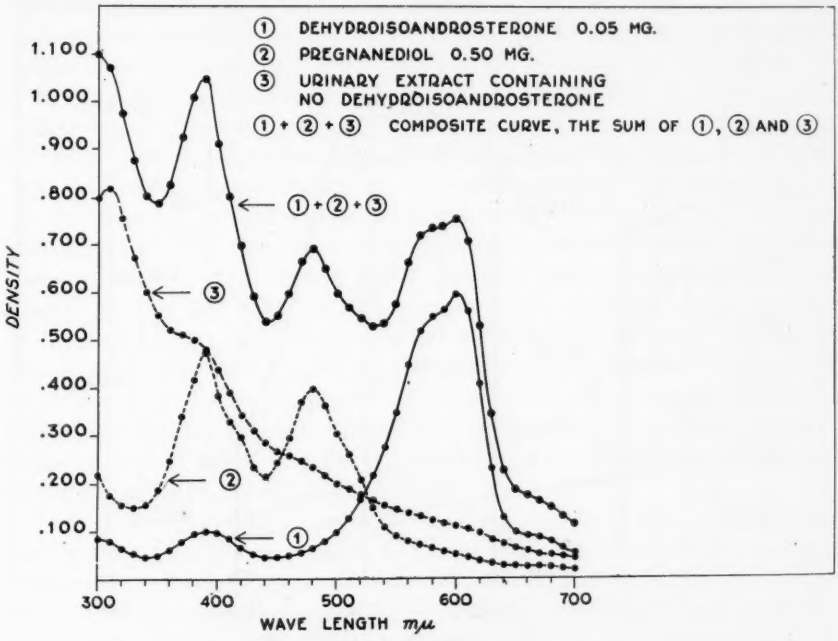

FIGURE 1

was used. Since this formula has been found very practical and useful the derivation seems to be worthy of publication.

In Figure 1 the absorption curves are given for 50 micrograms of pure dehydroisoandrosterone, 500 micrograms of pregnanediol and a butyl alcohol extract of the urine of a patient following removal of an adrenal tumor. The color was developed by the method previously described (4), *i.e.*, by heating the dry residue of a urinary extract for twelve minutes at 55 degrees with 2 cubic centimeters of the alcohol-sulfuric acid reagent, cooling for one minute, and then adding 3 cubic centimeters of 95 per cent ethyl alcohol. All absorption curves were made on 5 cubic centimeter volumes measured in the Beckman spectrophotometer, using square cuvettes with a depth of 1 centimeter. These three absorption curves were chosen, because

one contains virtually no steroid and is in a sense a urinary blank; another is produced by a compound, pregnanediol, which is known to be present in the urine during pregnancy but which shows no absorption maximum at 600 mμ; and the third, dehydroisoandrosterone, which gives the specific purple color with an absorption maximum at 600 mμ. It is obvious that the absorption curves for the pregnanediol and the urinary blank closely approach straight lines in the region where dehydroisoandrosterone exhibits

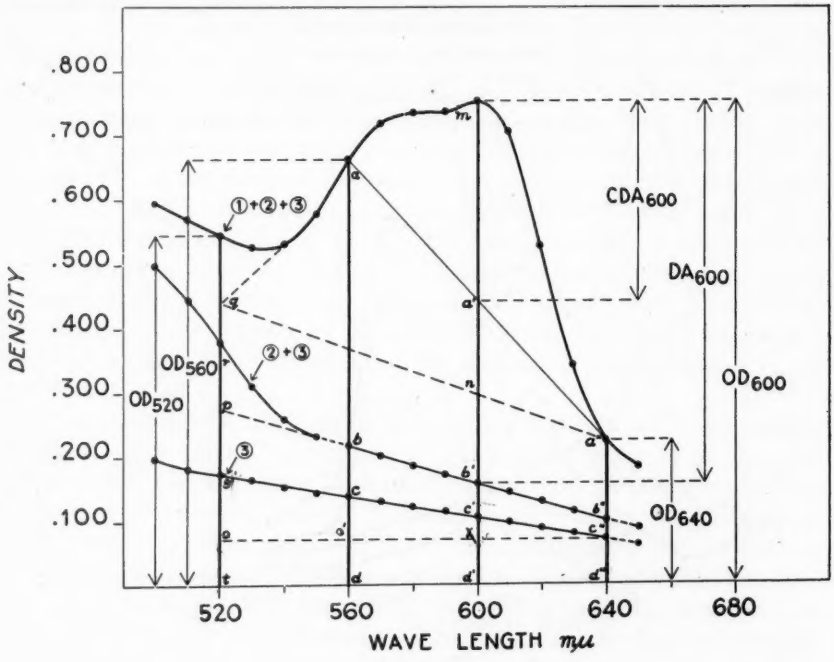

FIGURE 2

maximum absorption. When these two compounds and the urine blank are mixed in the same proportions, a composite curve is obtained which is the sum of the curves produced by each of the three components, assuming of course, that the reaction of the three when pooled with the reagent is the same as when carried out individually. This composite curve closely resembles curves obtained in actual study of the steroid excretion in certain cases of adrenal tumor and hence is one suitable for use in illustrating the general method of derivation of the equations.

Figure 2 is a larger scale reproduction of Figure 1, showing the portion of the absorption curves for the analysis. The data are rearranged so that the absorption curves are individually added. Curve 3 is the same as in Figure 1. The next curve represents the sum of curves 2 and 3 and hence

shows the total amount of optical density produced by the contaminating substances. The uppermost curve is the sum of curves 1, 2, and 3. Rearrangement in this manner shows more clearly the individual contribution of each substance to the total density. The area below curve 3 indicates the density due to the urinary extract; the area between this and curve 2 +3 indicates the density due to 500 micrograms of pregnanediol and the area between curve 2+3 and curve 1+2+3, gives the density due to 50 micrograms of dehydroisoandrosterone. This last area is, of course, the same as the area beneath the absorption curve produced by 50 micrograms of dehydroisoandrosterone and plotted in Figure 1.

In the actual analysis of an unknown curve the problem is to determine what portion of the observed optical density at 600 mμ (OD_{600}) is produced by dehydroisoandrosterone (DA_{600}). In this case the $OD_{600}=.750$, the $DA_{600}=.592$ and the density of the two impurities combined (DI_{600}) $=.158$. The values which are obtainable in an unknown mixture are the OD_{600}, and likewise the observed densities at any other wave lengths which seem to be indicated. In the figure, the four heavy perpendicular lines erected at 520, 560, 600 and 640, represent the observed densities (OD) at these points. At each of these points the observed density is the sum of the densities due to the three ingredients of the mixture. It is necessary, therefore, to find some means of subtracting from the compositive curve the portion due to the impurities or, to state the problem more practically, some method of predicting the point b′ so that the density due to the impurities at 600 (DI_{600}) can be determined.

First, a straight line is drawn connecting s and c.'' This follows very closely the actual points on curve 3. A line is then drawn parallel to the base from c,'' intersecting line st at o and c′d′ at x. This divides the area below curve 3 and between the perpendicular lines at 520 and 640 into an upper right angle triangle and a lower rectangle. Now let the perpendicular at 600 be located in the general case, x distance from the 520 perpendicular but lying between 520 and 640.

In the triangle soc''

$$c'x/so=c''x/c''o \qquad c'x=\frac{c''x}{c''o}so$$

but so+ot=density due to the impurity (urinary extract) at 520 (DI_{520})

c''d''=ot=density due to the impurity at 640 (DI_{640})

$$so=DI_{520}-DI_{640}$$

$$c'x=\frac{c''x}{c''o}(DI_{520}-DI_{640})$$

$$c'x+d'x=\frac{c''x}{c''o}(DI_{520}-DI_{640})+d'x.$$

In this equation, however,

$c'x+d'x=DI_x$ (density due to the impurity at wave length x; in the diagram, x is at 600)

$d'x=DI_{640}$

$$\text{therefore} \quad DI_x=\frac{c''x}{c''o}(DI_{520}-DI_{640})+DI_{640} \tag{I}$$

This equation is valuable since it shows that the density of the impurity (DI) at wave length x, located between the other wave lengths can be predicted from the densities at the other two wave lengths. In this specific case

$$DI_{600}=\frac{640-600}{640-520}(.175-.072)+.072$$

$$DI_{600}=\frac{40}{120}(.103)+.072$$

$DI_{600}=.034+.072=.106$ (the observed value is .108).

In an even more general sense this equation is applicable to the analysis of any absorption curve which is the sum of any number of curves, providing the sum of these curves is a straight line. However, inspection of the absorption curve 2+3 shows that a straight line drawn from r to b″ would cross the perpendicular at 600 considerably above the point b″. Therefore, in this particular case, selection of the densities at 520, 600 and 640 for analysis does not lead to an accurate prediction of the DI_{600} but gives a value too great (actually .195 instead of .160). The calculated value for the density due to dehydroisoandrosterone at 600 (DA_{600}) would be correspondingly low. In Figure 2, however, if 560, 600 and 640 are chosen for the points for analysis, curve 2+3 becomes a straight line. This serves to simplify equation I considerably, for then

$$\frac{c''x'}{c''o'}=1/2$$

and

$$DI_{600}=1/2(DI_{560}-DI_{640})+DI_{640}$$

$$DI_{600}=\frac{DI_{560}+DI_{640}}{2}. \tag{II}$$

This equation merely says that the density at a point midway between two other points is equal to one half the sum of the densities at the other points. In this case, analyzing curve 2+3

$$DI_{600}=\frac{.216+.099}{2}=.157 \text{ (observed value is .158).}$$

This equation II is not, however, directly utilizable because at no point is the observed density (OD) made up entirely of density due either to the impurities (DI) or to the dehydroisoandrosterone (DA). Actually the observed density is composed of two components (DI and DA).

$$DA_{600} = OD_{600} - DI_{600}$$

From equation (II), $$DA_{600} = OD_{600} - \frac{DI_{560} + DI_{640}}{2} . \qquad \text{(III)}$$

This equation is more helpful because it contains densities at the two other points. It still cannot be used directly because the DI_{560} and the DI_{640} are not actually known. They contribute to the OD_{560} and the OD_{640}. The remainder of the observed densities at these two points is due entirely to dehydroisoandrosterone. Equation III is readily transformed, however, by subtracting the following expression from each side

$$\frac{DA_{560} + DA_{640}}{2} .$$

This expression is represented in Figure 2 by the line a′b′. Therefore,

$$DA_{600} - \frac{DA_{560} + DA_{640}}{2} = OD_{600} - \frac{DI_{560} + DI_{640}}{2} - \frac{DA_{560} + DA_{640}}{2}$$

$$DA_{600} - \frac{DA_{560} + DA_{640}}{2} = OD_{600} - \frac{(DI_{560} + DA_{560}) + (DI_{640} + DA_{640})}{2}$$

but

$$DI_{560} + DA_{560} = OD_{560}$$
$$DI_{640} + DA_{640} = OD_{640}$$

therefore, $$DA_{600} - \frac{DA_{560} + DA_{640}}{2} = OD_{600} - \frac{OD_{560} + OD_{640}}{2} . \qquad \text{(IV)}$$

The members of the right side of the equation are always obtainable from the spectral transmission curve of the unknown mixture being examined. The members of the left side of the equation are all readily obtained by study of the transmission curves of pure dehydroisoandrosterone. The expression on the left is designated as CDA_{600} (calculated density due to dehydroisoandrosterone at 600). Simple inspection shows now that the CDA_{600} represents only the density of the peak above line a a′ a″, this being due entirely to dehydroisoandrosterone. The remainder of the OD_{600} is made up of the impurities together with a portion of the total density due to dehydroisoandrosterone.

The final equation used in the analysis of these spectral transmission curves, then, is

$$CDA_{600} = OD_{600} - \frac{OD_{560} + OD_{640}}{2}. \qquad (V)$$

This merely states that a definite part of the density at a given wave length due to a certain compound having an absorption maximum at that point can be obtained by subtracting from the observed density at that point one half the sum of the observed densities at two other points equidistant from the absorption maximum. The equation is generally applicable to the analysis of any absorption curve, providing the sum total of the spectral densities produced by the contaminating substances gives essentially a straight line.

It should be pointed out that equation IV contains none of the constants ordinarily used in the analysis of mixed absorptions curves. The equation does, however, contain a member, $\frac{DA_{560} + DA_{640}}{2}$, which varies directly with the total absorption of the compound being determined. In actual practice the wave lengths used should be chosen so that this factor is kept as small as possible within the other limitations of the method, namely, that the absorption curve produced by the impurities be linear. In the particular example chosen, the values for pure dehydroisoandrosterone are .592 at 600, .445 at 560 and .125 at 640, and the observed densities for the mixture are .750 at 600, .662 at 560 and .225 at 640. When these are substituted in the equation

$$.592 - \frac{.445 + .125}{2} = .750 - \frac{.662 + .225}{2}$$

$$.592 - .285 = .750 - .443$$

$$.307 = .307.$$

This method seems to discard a certain portion of the absorption produced by the compound being measured. The first impression is that this can be avoided by introducing the constants customarily used. Simple rearrangements of equation IV, however, show that the introduction of other constants does nothing other than to complicate the arithmetic.

Let

$$\frac{DA_{560}}{DA_{600}} = k_1 \quad \text{and} \quad \frac{DA_{640}}{DA_{600}} = k_2.$$

Divide equation IV by DA_{600}
then

$$\frac{2DA_{600}-(DA_{560}+DA_{640})}{DA_{600}}=\frac{2OD_{600}-(OD_{560}+OD_{640})}{DA_{600}}$$

$$DA_{600}=\frac{OD_{600}-\frac{OD_{560}+OD_{640}}{2}}{1-\frac{(k_1+k_2)}{2}}. \qquad \text{(VI)}$$

This equation gives the total density produced by the compound being measured, dehydroisoandrosterone, at the maximum (DA_{600}) in terms of the observed densities at the specified wave lengths, together with the necessary constants. Substitution of the actual values shows that the some proportionate amount of the total density which is seemingly discarded is actually accounted for by division rather than by addition. In equation VI the proper value for DA_{600} is obtained by dividing the CDA_{600} by a constant whereas, when using equation IV, DA_{600} is obtained by adding an amount which, although not a constant, nevertheless varies directly with the value for DA_{600}.

In order to use the equation it is necessary to set up a standard curve showing the relationship between density and amount. With dehydroisoandrosterone, for example, absorption curves were obtained with various amounts between 5 and 100 micrograms, the color being developed by the method previously described. Such curves are shown in Figure 3, a total of 80 determinations being made using various amounts of dehydroisoandrosterone. The uppermost line shows the relationship between density and amount when the total density at 600 (DA_{600}) is plotted. The lowermost curve shows the relationship when the CDA_{600} is plotted using the three wave lengths 560, 600 and 640 for the calculations. The actual amount of dehydroisoandrosterone can be readily determined from the graph or by using the equation for the straight line. The difference in the slopes of the uppermost and lowermost curves shows that nearly half of the density produced by the dehydroisoandrosterone is included in the density produced by the impurities when the narrower band is used in the calculations.

At first sight, there may seem to be no preference between equations IV and VI but in actual use equation IV is simpler. Both equations inevitably give zero as an answer when no absorption maximum is present. There is a valuable difference however. Equation IV contains no constants in the right side. This actually means that any compound, unknown in

chemical structure and not available, which gives an absorption band can be quantitatively measured, provided the interfering absorption curve is linear. A certain definite but unknown per cent of the total density at the maximum is discarded in the calculation until such time as the pure substance becomes available so that the left side of the equation can be accurately determined. This fact makes equation IV of general usefulness in following unknown compounds through various schemes of separation, since its use makes it possible to measure in units of density the amount present in a given fraction.

Finally, is it possible to work out a general solution of the problem in such a way that no constants derived from a study of the pure substance enter into the calculations, and without restricting the problem so that the two wave lengths on either side of the maximum are equidistant from the maximum? In Figure 2, if wave lengths 520 and 640 are chosen, the linear nature of the DI does not exist. However, if the line bb'b'' be projected it will cross the perpendicular at p (DI = .275). The OD_{520} then becomes .438. ($DA_{520} = .163$; $DI_{520} = .275$). In this hypothetical case, the area below the line pb'' is produced by the impurity, the area between qa'' and pb'' represents the area due to dehydroisoandrosterone which is included with the impurity, and the line mn represents the calculated density due to DA. As in the development of equation IV, let the perpendicular line at 600 be x distance from the perpendicular line at 520 but between 520 and 640. Then

$$OD_x = mn + nb' + b'd'$$

$$nb' = \frac{c''x}{c''o}(DA_{520} - DA_{640}) + DA_{640}$$

$$b'd' = \frac{c''x}{c''o}(DI_{520} - DI_{640}) + DI_{640}.$$

Let

$$mn = CDA_x \quad \text{and} \quad \frac{c''x}{c''o} = K_1$$

$$CDA_x = OD_x - [K_1(DI_{520} - DI_{640}) + DI_{640}] - [K_1(DA_{520} - DA_{640}) + DA_{640}]$$

$$CDA_x = OD_x - K_1(DI_{520} + DA_{520}) + K_1(DI_{640} + DA_{640}) - (DI_{640} + DA_{640})$$

$$CDA_x = OD_x - K_1OD_{520} + K_1OD_{640} - OD_{640}. \qquad \text{(VII)}$$

But

$$CDA_x = DA_x - K_1DA_{520} + K_1DA_{640} - DA_{640}.$$

Equation VII is readily used after determining the values for CDA_x for various amounts of dehydroisoandrosterone. The linear relation be-

tween this equation and the amount of dehydroisoandrosterone is shown in Figure 3. The slope of this line more nearly approaches that of the uppermost line than does the slope of the lowermost line. In the diagram this is obvious. The DA_{600} for 50 μg is represented by the line mb', the CDA_{600} by the line ma' when equation IV is used and the CDA_{600} by the line mn when equation VII is used.

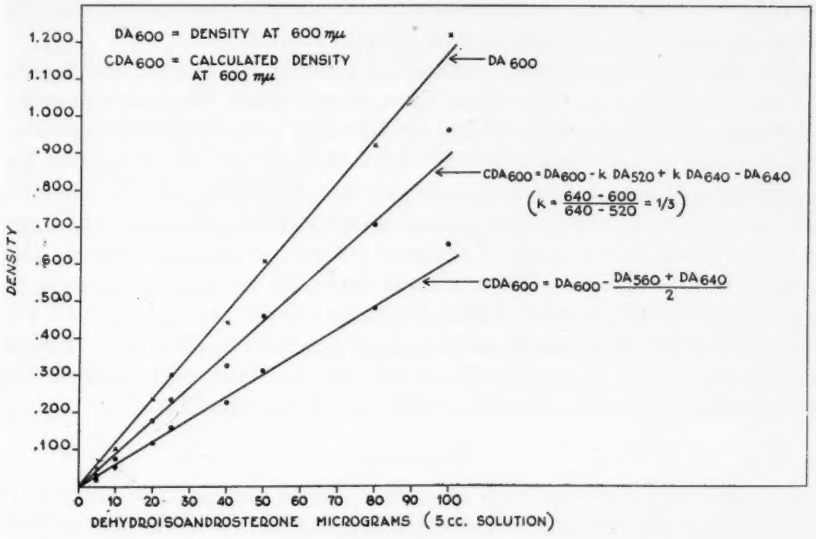

FIGURE 3

Finally, equation VII provides the mathematical proof for an extremely simple method of analyzing complicated absorption curves, which utilizes only a ruler and simple arithmetic. The calculated density (CDA_x in this special case) is the length of the line mn. The actual value of this line is determined by comparing it with the actual value for a similar line obtained from similar treatment of the absorption curve of pure dehydroisoandrosterone by the same method. Therefore, in actual practice it is only necessary to plot the transmission curve of the unknown and then draw a straight line between the densities at any two wave lengths on either side of the maximum and measure that portion of the total density at the maximum which is above the straight line. (In the diagram, aa'a", and qna" are such straight lines.) The transmission curve of a known amount of the pure substance is plotted and a straight line is drawn between the densities at the same two wave lengths on either side of the maximum. The portion of the total density at the maximum which lies above the straight line is then determined. By simple proprotion, the density of the unknown above the straight line is compared with the density above the

straight line of a known amount of the pure compound. This simple procedure is made possible, of course, because of the particular natures of the equations in which all the values on the left side are obtained from the pure substance and all the values on the right from the unknown.

In actual practice there is probably little need for using any equation other than equation V. The colorimetric methods for measuring urinary steroids are subject to so much error that the slight advantage obtained by using equation VII is insufficient to warrant its use.

A similar solution has been worked out recently by Morton and Stubbs (5), likewise using measurements at three wave lengths. They also, to seek a simplification, restrict the problem by selecting wave lengths situated on either side of the maximum so that the ratio between the density at the maximum and the density at the selected lateral wave lengths is constant. Also the equations which they have developed utilize constants which can be obtained only by a study of the pure compound being measured. This is not a serious objection since in most cases the compound being determined will be known and available for study. However, equations IV and VII seem to be just as simple and are more generally applicable because of the elimination of all constants, except the constant pertaining to the particular wave lengths, from the right side of the equation.

SUMMARY

A simple equation has been developed which permits the analysis of mixed absorption curves provided the absorption curves of the contaminating substances closely approximate a straight line. This is found to be the case in the study of urinary extracts for estrogen and dehydroisoandrosterone by modifications of the Kober test.

The equation used is

$$CDX_x = OD_x - \frac{OD_a + OD_b}{2}$$

where:

OD_x = the observed density at wave length x, at the absorption maximum.

OD_a and OD_b = the observed densities at wave lengths a and b; a and b must be equidistant from x.

CDX_x = calculated density due to substance X having an absorption maximum at wave length x. This represents only a part of the total density at wave length x due to compound X, but the actual amount of X is readily calculated from CDX_x.

The general solution is given also where the other two wave lengths are not equidistant from wave length x.

REFERENCES

1. KOBER, S.: Ein kolorimetrische Bestimmung des Brunsthormons (Menformon) *Biochem. Ztschr.* **239:** 209, 1931.
2. ZIMMERMANN, W.: Colorimetrische Bestimmung der Keimdrüsenhormone, *Ztschr. f. physiol. Chem.* **245:** 47, 1936.
3. GIBSON, J. G., and EVANS W. A.,: Clinical studies of blood volume; clinical application of method employing azo dye "Evans blue" and spectrophotometer, *J. Clin. Investigation* **16:** 301, 1937.
4. ALLEN, W. M.; HAYWARD, S. J.,, and PINTO, A.: A color test for dehydroisoandrosterone and closely related steroids, of use in the diagnosis of adrenocortical tumors, *J. Clin. Endocrinol.* **10:** 54, 1950.
5. MORTON, R. A., and STUBBS, A. L.: Photoelectric spectrophotometry applied to the analysis of mixtures and vitamin A oils, *Analyst* **71:** 348, 1946.

COMPARISON OF CHEMICAL AND BIOLOGIC ASSAY OF CORTIN-LIKE SUBSTANCES IN NORMAL MALE URINE*

ARNE N. WICK, PH.D., ELDEN PECKA, JR., B.S. AND ROBERT MEDZ, M.S.

From The Scripps Metabolic Clinic, La Jolla, California

CHEMICAL analysis for the estimation of urinary "cortin" excretion in normal and abnormal subjects has been widely employed recently. The chemical methods have been based on oxidation of the α-ketol side chain at carbon 17 and the α, β-unsaturated 3-ketone grouping of ring A of the adrenal steroid nucleus (1, 2, 3, 4, 5). Both groups are essential for biologic cortin activity and chemically have a strong reducing action. The exact nature of the urinary material is not known although it has been assumed that it represents one or more of the known biologically active compounds which have been isolated from adrenal cortical extracts. This possibility has been further strengthened by the recent work of Mason *et al.* (6) who isolated 17-hydroxycorticosterone from the urine in a case of Cushing's syndrome associated with severe diabetes mellitus.

Since the chemical methods of estimation of urinary cortin are sufficiently simple to be useful in routine clinical and research studies, it was considered advisable to correlate the sensitive but less specific chemical method with a biologic procedure. In addition, information regarding the relationship of the two methods was desirable from the standpoint of using the chemical method for isolation studies.

METHODS

The chemical procedure used in these studies is similar to that of Lowenstein, Corcoran and Page (3) in which formaldehyde is formed by oxidation with periodic acid. The detailed procedure was obtained from Harold L. Mason of the Mayo Clinic in the summer of 1947. A method which is approximately the same as this one has been described more recently by Daughaday, Jaffe and Williams (5). In our work the formaldehyde was distilled prior to the color development. The intensity was determined at wave length 570 mμ in a Beckman model Du spectrophotometer. Corticos-

Received for publication June 16, 1949.

* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

TABLE 1. COMPARISON OF CORTIN-LIKE MATERIAL IN FRACTIONATED NORMAL MALE URINE AS DETERMINED BY A CHEMICAL AND BY A BIOLOGIC PROCEDURE

Experiment No.	Total vol. urine extracted, (liters)	Fraction	Weight of fraction (Gm.)	Chemical analysis: Corticosterone equivalent Gm.	Chemical analysis: Corticosterone equivalent %	Biologic assay: Corticosterone equivalent admin., mg./rat*	Biologic assay: Liver glycogen,** %
1	240	Neutral extract	0.587	0.074	13		
		Ketonic (pH 2)	0.094	0.020	21	0.5; 1.0	0.09; 0.07
		Nonketonic	0.235	0.010	4		
						1 mg. pure corticosterone	0.97
						Controls	0.05
2	850	Neutral extract	2.899				
		Ketonic (pH 3)	0.457	0.009	2	0.9	0.05
		Ketonic (pH 0.6)	0.208	0.045	22	4; 1	0.40; 0.06
		Nonketonic	1.502	0.142	10	5	0.05
						0.5 mg. pure corticosterone	0.39
						Controls	0.05
3	490	Neutral extract	1.483	0.096	7		
		Ketonic (pH 2.3)	0.178	0.048	27	3	0.34
		Ketonic (pH 0.5)	0.059	0.014	24	1	0.12
		Nonketonic	1.070	0.048	5		
						0.5 mg. pure corticosterone	0.46
						Controls	0.06

* "Corticosterone equivalent" is the apparent concentration of corticosterone as determined by chemical analysis.

** Calculated on basis of wet weight of liver.

terone was used as the reference standard and the results are consequently expressed in terms of corticosterone.

The bio-assay method chosen was based on the glycogen deposition in adrenalectomized rats as carried out by Olson *et al.* (7) and modified by Pabst and co-workers (8). The test substances were used in oil solution and administered to the animals at various concentrations referred to as

"corticosterone equivalent," being the value determined by chemical analysis. For purposes of comparison the urine extracts were assayed simultaneously with 2 groups of animals, one group receiving corticosterone and the other serving as controls. There was a minimum of 6 rats in each group. The results given in Table 1 are the average for the group.

EXPERIMENTAL

The urine was collected from men students daily, in the rest rooms at San Diego State College. It represents the output of day urine from men students in the 18 to 25-year age group. Each day after the collection, the urine was clarified by filtration through a bed of filter aid (Celite 545, Johns-Manville Corp.). For 100 liters of clarified urine (pH 5.0) the following steps were used:

Two extractions were carried out with a half volume of ethylene dichloride. Each extraction was mixed thirty minutes in a glass-lined tank equipped with a high speed stirrer. The emulsion was reduced approximately 90 per cent by passing the extraction mixture through a two to three-inch layer of filter aid. The small amount of emulsion remaining after the filtration was readily dispersed by freezing the mixture to −20°C. with powdered carbon dioxide and filtering off the resulting ice particles. The clear ethylene dichloride extract was concentrated to 400 ml. in a vacuum stainless steel flash evaporator. The temperature of the vapors in the flash chamber was well below 15°C. The ethylene dichloride concentrate was vigorously extracted nine times with 200 ml. portions of water. The resulting water extract which contained the formaldehyde-producing material was re-extracted five times with 300 ml. portions of chloroform. The chloroform extract was washed with cold saturated solution of potassium bicarbonate and then with water. All wash solutions were backwashed so as to minimize loss of neutral solids. The chloroform solution, after drying over anhydrous magnesium sulphate, was concentrated to dryness in vacuum under nitrogen to give a residue referred to as "neutral extract." This residue was separated into ketonic and nonketonic fractions with Girard's reagent T as used by Talbot *et al.* (2).

The results obtained from extracting 3 lots of urine are shown in Table 1.

DISCUSSION

It is observed in experiment 1 that the neutral extract from 240 liters of urine contains a considerable quantity of formaldehydogenic material. The ketonic fraction assaying 21 per cent corticosterone by the chemical method gave no glycogen increase above the control value when tested at the corticosterone equivalent of 0.5 and 1.0 mg./rat. On the other hand, the group receiving 1 mg./rat of pure corticosterone gave the substantial liver glycogen value of 0.97 per cent.

In the second experiment the processing of 850 liters of urine gave a neutral extract weighing 2.899 grams. Two ketonic fractions were obtained by progressive acidification and extraction after the application of Girard's reagent T. The first ketonic fraction (pH 2–3) was obtained after a two-

hour room temperature hydrolysis period and the second ketonic sample (pH 0.5–0.6) was obtained by further acidification and permitting the mixture to hydrolyze at room temperature twelve hours before extracting with chloroform. Referring to Table 1 and the biologic assays, it is seen that the second ketonic fraction obtained at pH 0.6 gave a glycogen value corresponding to 0.5 mg. of pure corticosterone only when assayed at a level of 4 mg. corticosterone equivalent per rat. The first ketonic fraction (pH 3) failed to elicit any glycogen response when assayed at a level of 0.9 mg. corticosterone equivalent per rat. Sufficient material was not available for re-assays at higher levels. Bioassay of the nonketonic fraction at 5 mg. corticosterone equivalent (total solids 50 mg./rat) indicated the absence of glycogenic properties.

The results for the third experiment confirm the previous finding that the nonketonic fraction contains considerable amounts of formaldehyde producing substances which are biologically inactive. The biologic assays of the ketonic fraction (pH 2.3) indicated that the 3 mg. corticosterone equivalent (total solids 11 mg./rat) was comparable to that obtained by 0.5 mg. pure corticosterone.

From these results it is concluded that chemical analysis of fractionated normal urine extracts based on the formation of formaldehyde by periodic acid oxidation, gives values in terms of corticosterone 6 to 8 times higher than those obtained by in vivo glycogen deposition. The concentration of the glycogenic substances in normal male urine is estimated from our results to be 10 to 20 gammas per liter of urine expressed as "corticosterone equivalent." These values are approximately one half that reported by Heard *et al.* (4) after taking into consideration the difference in biologic titer of the two standards. The higher urinary cortin values obtained by these workers may be partially explained by their extraction of the urine after acidification.

Although chemical analysis gives high results in comparison with the glycogen deposition assay, with normal male urine, it is likely that a closer relationship may exist in female or in abnormal urine. The increase of cortin-like substances, which was observed by Mason *et al.* (9) and Forsham and co-workers (10), after the administration of the anterior pituitary adrenocorticotropic hormone to normal subjects undoubtedly reflects a true stimulation of adrenocortical function.

SUMMARY

A comparison of chemical and biologic methods for determining the cortin-like substances in normal male urine has been made. It was found that the chemical colorimetric procedure gives values 6 to 8 times higher than those obtained by the glycogen deposition assay. It is suggested that the two methods may give more comparable results with abnormal urine.

REFERENCES

1. Fieser, L. F.; Fields, M., and Lieberman, S.: Concerning the characterization of possible cortical hormone metabolites in urine, *J. Biol. Chem.* **156:** 191–202 (Nov.) 1944.
2. Talbot, N. B.; Saltzman, A. H.; Wixom, R. L., and Wolfe, J. K.: The colorimetric assay of urinary corticosteroid-like substances, *J. Biol. Chem.* **160:** 535–546 (Oct.) 1945.
3. Lowenstein, B. E.; Corcoran, A. C., and Page, I. H.: Determination of corticosteroids in urine, (abstract) *Endocrinology* **39:** 82 (July) 1946.
4. Heard, R. D. H.; Sobel, H., and Venning, E. H.: The neutral lipide-soluble reducing substances of urine as an index of adrenal cortical function, *J. Biol. Chem.* **165:** 699–710 (Oct.) 1946.
5. Daughaday, W. H.; Jaffe, H., and Williams, R. H.: Chemical assay for "cortin," *J. Clin. Endocrinol.* **8:** 166–174 (Feb.) 1948.
6. Mason, H. L., and Sprague, R. G.: Isolation of 17-hydroxycorticosterone from the urine in a case of Cushing's syndrome associated with severe diabetes mellitus, *J. Biol. Chem.* **175:** 451–456 (Aug.) 1948.
7. Olson, R. E.; Jacobs, F. A.; Richert, D.; Thayer, S. A.; Kopp, L. J., and Wade, N. J.: The comparative bioassay of several extracts of the adrenal cortex in tests employing four separate physiological responses, *Endocrinology* **35:** 430–455 (Dec.) 1944.
8. Pabst, M. L.; Sheppard, R., and Kuizenga, M. H.: Comparison of liver-glycogen deposition and work performance tests for the bioassay of adrenal cortex hormone, *Endocrinology* **41:** 55–65 (July) 1947.
9. Mason, H. L.; Power, M. H.; Rynearson, E. H.; Ciaramelli, L. C.; Li, Choh Hao, and Evans, H. M.: Results of administration of anterior pituitary adrenocorticotropic hormone to a normal human subject, *J. Clin. Endocrinol.* **8:** 1–14 (Jan.) 1948.
10. Forsham, P. H.; Thorn, G. W.; Prunty, F. T. G., and Hills, A. G.: Clinical studies with pituitary adrenocorticotropin, *J. Clin. Endocrinol.* **8:** 15–66 (Jan.) 1948.

PREGNANEDIOL EXCRETION IN NORMAL WOMEN*

JOSEPH ROGERS, M.D.† AND SOMERS H. STURGIS, M.D.‡

From the Vincent Memorial Laboratory of The Vincent Memorial Hospital affiliated with The Massachusetts General Hospital, Boston, Massachusetts

MANY previous workers (1–7) have reported observations on the urinary excretion of pregnanediol during the normal menstrual cycle. Most of the results recorded have been obtained by the method of Venning (8, 9) which isolates pregnanediol as the complex, sodium pregnanediol glucuronidate. Difficulties with the Venning method have led to the development of more sensitive, reliable procedures which isolate pregnanediol in the free state. Relatively few observations on pregnanediol excretion in the normal menstrual cycle have been made by such methods (10, 11). It is the purpose of this paper to present further measurements of the daily excretion of pregnanediol determined in the free state in normal menstrual cycles correlated with the basal body temperature and to discuss the significance of pregnanediol excretion.

METHODS

The pregnanediol determinations were done by a modification developed by Astwood, Grady, and Wermer (12) of the method reported by Astwood and Jones in 1941 (13). The details of the procedure are as follows:

A twenty-four hour urine specimen was obtained. If the volume was more than 1500 cc., an aliquot was used.

The volume of urine was measured and poured into a round-bottom flask, 100 cc. of toluene was added and then 1 cc. of 18 N sulfuric acid for each 10 cc. of urine. The flask was attached to a reflux condenser and the contents boiled for fifteen minutes. The flame was removed and the flask allowed to cool with gentle shaking until boiling ceased. The ingredients were poured into a separating funnel, shaken, and the toluene extract allowed to separate before discarding the urine. The toluene extract was washed twice with 2 N sodium hydroxide and twice with hot distilled water (50 cc. each time). The extract was poured into an Erlenmeyer flask and excess water was boiled off on a hot plate. A column was made using aluminum oxide ($6\frac{1}{2}$ grams of water/100 grams) about $\frac{3}{4}$ inch deep in a 2 cm. diameter cintered glass funnel. Twenty cc. of toluene was added, the aluminum was suspended by vigorous mixing with a spatula and then allowed to settle. The aluminum oxide was covered with a layer of sand, care being taken not to disturb the column. The toluene extract was poured into the funnel and drawn through by gentle suction.

Received for publication June 13, 1949.

* This study was supported in part by a grant from the American Cancer Society.

† Assistant Physician, New England Center Hospital: Instructor in Medicine, Tufts College Medical School.

‡ Clinical Associate in Gynecology, Harvard Medical School.

After the extract had run through, the column was washed with 10 cc. of toluene. Blue and green fractions will come through with the toluene. The column was washed with 0.5 per cent absolute alcohol in toluene until all the pink color was washed through. These fractions were discarded. Three per cent absolute alcohol in toluene was then used to remove the next fraction containing the pregnanediol, the color of which ranges from yellow to brown. The column was washed with approximately 100 cc. to be sure that all the pregnanediol was removed. (A slight brown color remains in the column but does not contain pregnanediol.) The extract was evaporated to dryness and transferred by means of a medicine dropper to a test tube with 95 per cent alcohol. This was evaporated to a measured volume of 1–4 cc., depending upon the amount of pregnanediol expected, and exactly 3 volumes of water were added slowly while heating. This solution was boiled gently and allowed to cool slowly. When dealing with small quantities of pregnanediol, as in menstrual cycles, the solution was allowed to stand at room temperature for twenty-four hours in order to obtain maximum precipitation. The precipitate was collected on a cintered glass funnel with gentle suction. If the precipitate was not white, it was redissolved in hot 95 per cent alcohol and reprecipitated and refiltered. When pure, the precipitate was dissolved in alcohol and transferred to a small previously weighed bottle, placed in the oven to dry, and weighed. Random specimens were checked for melting point, using a Fisher-Johns melting point apparatus.

Prior to making observations on the menstrual cycle, control data were obtained by adding known amounts of sodium pregnanediol glucuronidate[1] to urine from normal males (Table 1). The ratio of molecular weight of free

TABLE 1

Sodium pregnanediol glucuronidate added (mg.)	Theoretical yield (mg.)	Actual yield (mg.)	Per cent
25.3	11.9	4.9	43
19.4	8.7	5.4	62
15.3	6.8	2.9	85
14.8	6.6	3.8	64
4.2	1.87	1.7	94

pregnanediol to sodium pregnanediol glucuronidate .3 H_2O is 0.599 and sodium pregnanediol glucuronidate has been shown to be only about 80 per cent pure (14). A factor of .447 may therefore be used to calculate approximately the theoretical yield of free pregnanediol from known amounts of sodium pregnanediol glucuronidate. The percentage recovery of theoretical yield ranged from 43 to 94 per cent (Table 1).

Some of the pregnanediol yields to be reported were from twenty-four hour urine specimens forced through a collodion membrane in a Gorbman

[1] Generously supplied by Ayerst, McKenna & Harrison, Ltd.

ultrafilter (15) for purposes associated with another study. This procedure in some instances reduced the expected yield by about 15 to 20 per cent.

Quantities of pregnanediol below 0.6 mg. could not be weighed accurately but the presence of pure crystals with a satisfactory melting point, even though less than this amount, indicated qualitatively the presence of pregnanediol.

In order to determine the rate of excretion of pregnanediol in the urine after the administration of progesterone, 4 subjects were given 60 mg. of progesterone in aluminum monostearate[2] by intramuscular injection. The subjects were 2 normal males, a young woman in the eighth day of her menstrual cycle and a woman with amenorrhea.

Each of the subjects whose menstrual cycles were studied was a normal young woman with no clinical abnormalities of menstruation. Creatinine measurements were not done, as the women could be relied upon to collect the entire specimen. Each woman kept a daily record of her oral temperature taken each morning before arising.

RESULTS

As shown in Figure 1 the administration of 60 mg. of progesterone in aluminum monostearate resulted in the excretion of pregnanediol within the first twenty-four hours after injection. The amounts excreted ranged from 0.4 to 2.0 mg. and the total yield varied from 1.7 to 3.9 mg.

Determinations were made from day 8 of a normal menstrual cycle to day 29 (Fig. 2). Pregnanediol was not recovered till day 18 and then was obtained daily until the first day of the next menstrual flow. The temperature curve showed two points of rise and fall, the latter of which coincided with the first appearance of pregnanediol in the urine. Pregnanediol was first recovered one day after the temperature rise. The first appearance of pregnanediol preceded the onset of menstrual flow by eleven days. The daily amount recovered ranged from 0.4 to 2.1 mg. and the total yield for the cycle was 16.2 mg. In a second cycle, in the same subject (Fig. 3), pregnanediol in small amounts was recovered on day 14. None was found on day 15. Pregnanediol was found each day from day 16 to 29 except on day 20 when no collection was made. The first pregnanediol was recovered fifteen days prior to the onset of the next cycle and at a point when a temperature rise was occurring. The amount of pregnanediol recovered ranged from 0.1 to 2.8 mg. and the total amount excreted was 18.9 mg.

Figure 4 represents observations of pregnanediol excretion on another subject. Daily specimens were analyzed from day 7 to day 26 of the cycle. Throughout the entire cycle only small amounts were obtained, and the

[2] Generously supplied by Sharp and Dohme.

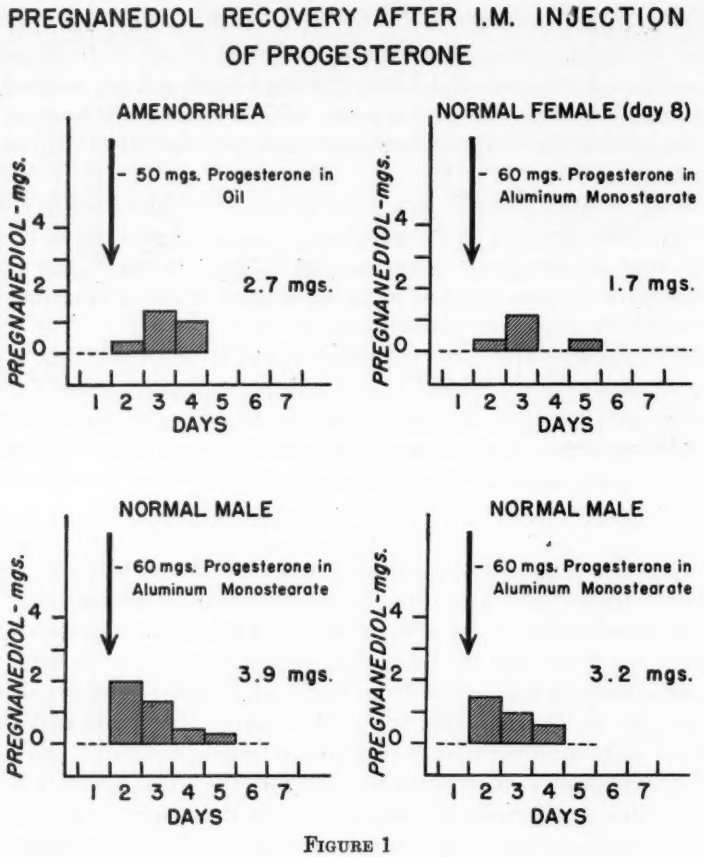

FIGURE 1

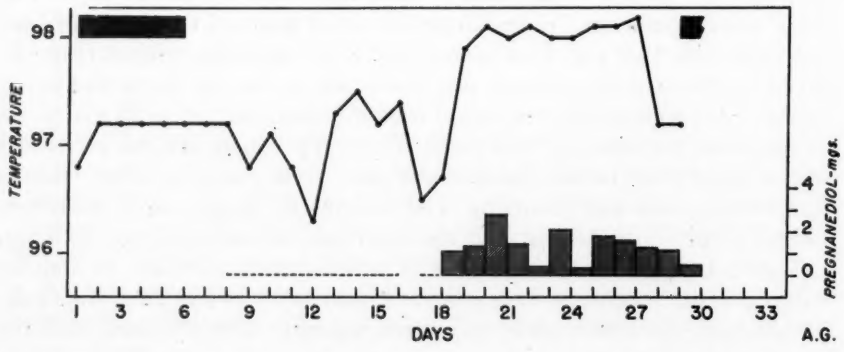

FIG. 2. First pregnanediol obtained on day after temperature rise. (In this and subsequent figures the black solid areas at the top of the graph represent the menstrual flow.)

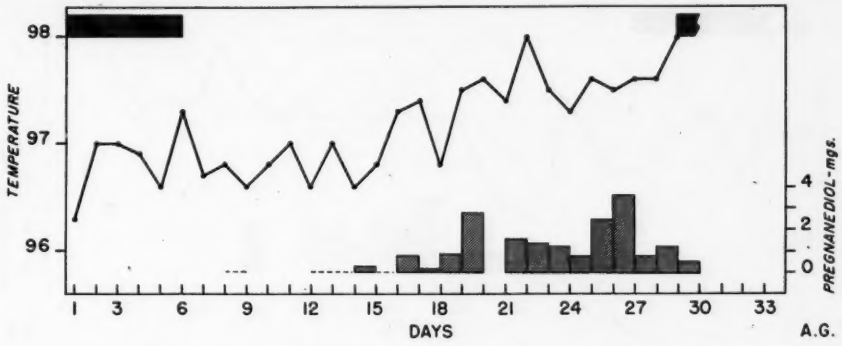

FIG. 3. First pregnanediol obtained on day of temperature rise.

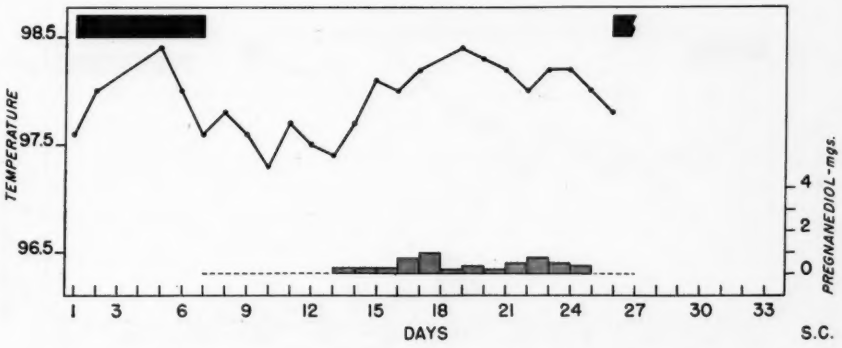

FIG. 4. First pregnanediol obtained on day of temperature rise.
(All specimens passed through Gorbman filter.)

daily yields ranged from 0.1 to 1.0 mg. with a total recovery of only 4.9 mg. The first recovery was on day 13. No pregnanediol was found on day 25, the day preceding the onset of flow. The temperature curve showed a rise coinciding with recovery of pregnanediol. Thirteen days elapsed from the first recovery of pregnanediol to the onset of flow.

In another subject (Fig. 5) the initial recovery of pregnanediol occurred just as the temperature was showing a rise, and preceded the onset of the next menstrual period by eighteen days. Collections were made from day 11 to 18 and a single specimen was obtained on day 24. The amounts recovered varied from 0.7 to 3.4 mg.

In the cycle shown in Figure 6 the initial pregnanediol was noted two days after a temperature rise had occurred and preceded the onset of the next menses by ten days. The amount recovered ranged from 0.5 to 2.0 mg.

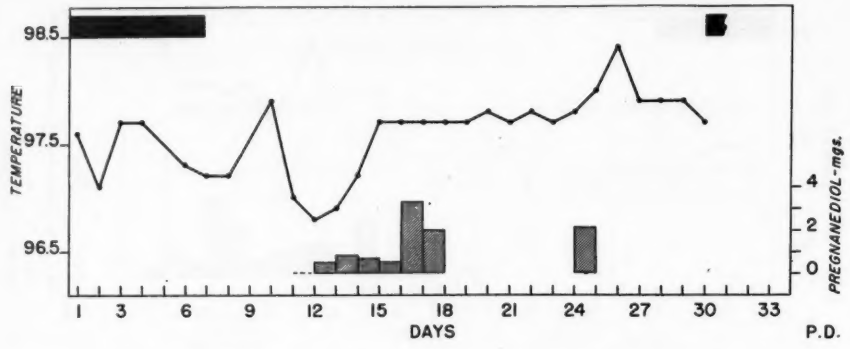

FIG. 5. First pregnanediol obtained on day of temperature rise.

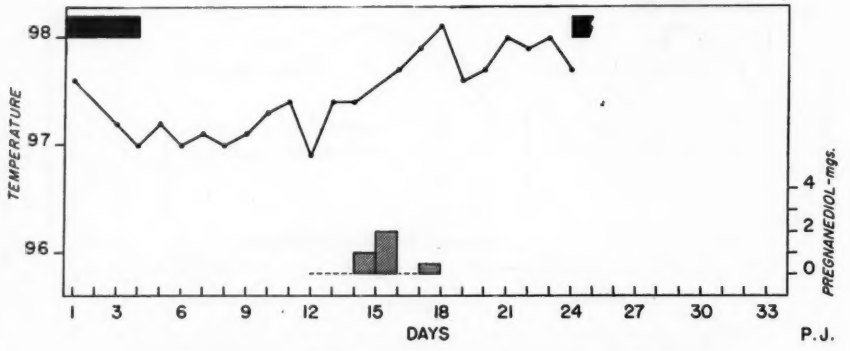

FIG. 6. First pregnanediol obtained two days after temperature rise.

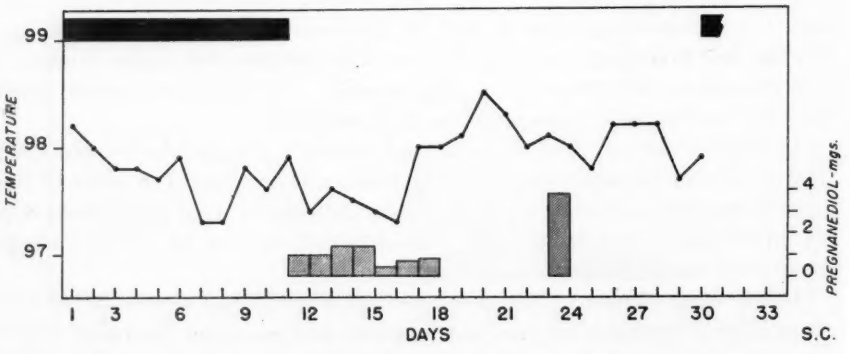

FIG. 7. First pregnanediol obtained five days before temperature rise and nineteen days before next catamenia.

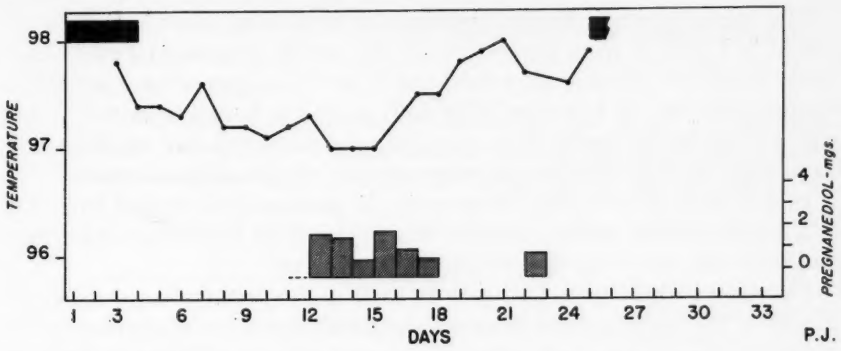

FIG. 8. Pregnanediol first obtained three days prior to onset of temperature rise.

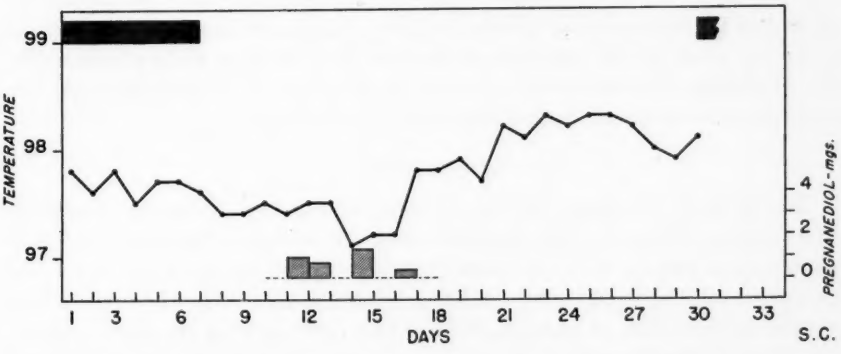

FIG. 9. Pregnanediol first obtained five days before temperature rise and nineteen days before onset of next catamenia.

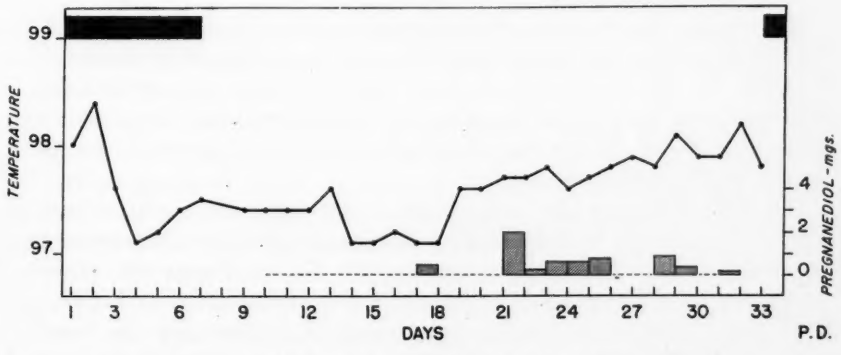

FIG. 10. Pregnanediol first obtained the day before temperature rise.

The values shown in Figure 7 represented a prior cycle in the same subject as in Figure 4, from days 11 to 17 and day 23. The first recovery was noted on day 11, one day after cessation of flow, and preceding a sustained temperature rise by five days. The daily amounts varied from 0.4 to 4.9 mg. A clear rise in temperature was not apparent until day 16. Nineteen days after the first recovery of pregnanediol, menstruation occurred.

In a fourth subject (Fig. 8) recovery of pregnanediol ranged from 0.9 to 2.2 mg. and the initial recovery was obtained as the temperature fell, and preceded the temperature rise by three days.

The observations made from day 10 to 16 of the next cycle are shown in Figure 9. The pregnanediol recovery antedated the rise in the temperature curve by five days and the onset of the next menstrual flow by nineteen days.

The initial appearance of pregnanediol during another cycle (Fig. 10) was noted on day 17 at a time when the temperature curve was at a low level and 16 days prior to the onset of flow. Daily specimens were analyzed from day 10 to 32 and negative results obtained on days 10 to 16, 18 to 20, 26, 27 and 31. The amounts recovered varied from 0.1 to 2.1 mgs. and the total amount excreted during the cycle was 5.7 mgs.

DISCUSSION

The finding of pregnanediol in the urine of a normal subject is generally accepted as evidence of the presence of progesterone in the body and progesterone is known to be produced by the corpus luteum. From the work of others and from our own evidence the administration of progesterone results in excretion of pregnanediol in the urine within the first twenty-four hours after injection. If endogenous progesterone is metabolized at the same rate as injected progesterone, these observations suggest that the presence of urinary pregnanediol may be a reliable indication that luteal tissue was functioning twenty-four hours previously.

Venning and Browne (1) and subsequent workers (2, 3, 4, 5) reported the disappearance of pregnanediol from the urine one to four days prior to menstruation. In only one of five normal cycles in which pregnanediol determinations were done up to the onset of the menstrual flow was such a disappearance noted. In the other four cycles pregnanediol was found up to the day of the menstrual flow.

The exact point on the temperature curve at which ovulation is supposed to occur is not known but most workers believe that the temperature rise is closely associated with ovulation (16, 17, 18). Farris (19) reported conception as taking place prior to any temperature change in 41 per cent of 27 cases. Whether or not the exact point on the temperature curve at which ovulation occurs can ever be determined, it has been demonstrated

by Davis and Fugo (20) that the elevation of the temperature occurring in the latter half of the menstrual cycle is related to the presence of progesterone in the body.

In the menstrual cycles observed during this study, no constant relationship between the temperature rise and the initial appearance of pregnanediol is apparent. This is seen in Table 2 which also indicates the cycle

TABLE 2. APPEARANCE TIME OF PREGNANEDIOL, CORRELATED WITH TEMPERATURE RISE

Subject	Figure No.	Length of menstrual cycle (days)	Correlation of pregnanediol appearance with temperature rise	Pregnanediol appearance (No. of days before catamenia)	Temperature rise (No. of days before catamenia)
S.C.	7	29	Five days before rise	19	14
S.C.	9	29	Five days before rise	19	14
P.J.	8	24	Three days before rise	13	10
P.D.	10	29	One day before rise	16	15
A.G.	3	28	Day of rise	15	15
S.C.	4	25	Day of rise	13	13
P.D.	5	29	Day of rise	18	18
A.G.	2	28	Day after rise	11	12
P.J.	6	23	Two days after rise	10	12

lengths and the number of days lapsing from the first appearance of pregnanediol, as well as the first rise in temperature and the onset of the next flow.

In each of these cycles the temperature readings of the latter half are higher than those of the first half and are consistent with temperature changes generally found in ovulatory cycles. Inasmuch as the rate of excretion of pregnanediol after injection of progesterone in 60 mg. doses seems constant, the appearance of pregnanediol at several different stages of the temperature curve suggests that the physiologic effect of rise in temperature produced by endogenous progesterone proceeds at a different rate than does the conversion of progesterone to pregnanediol and its excretion. The first appearance of pregnanediol in the urine may precede,

follow, or coincide with the temperature rise. Neither is the relation between temperature rise and pregnanediol excretion constant in the same individual in different cycles. Mack, Parks, and McDonald (11) on the other hand report consistent appearance of pregnanediol after presumptive ovulation as indicated by temperature rise.

The appearance of pregnanediol prior to a significant change in temperature, as shown in Figures 7, 8, and 9, raises the interesting question as to whether this phenomenon could represent progesterone secreted by luteinized theca prior to ovulation. In two of these cycles (Figs. 7 and 9), the initial appearance of pregnanediol preceded the next menstrual flow by nineteen days. In these instances, if pregnanediol excretion was indicative of the luteal phase, the phase was slightly prolonged. Davis and Fugo (20) noted pregnanediol prior to temperature rise but thought it was of extra-ovarian origin. Pregnanediol is, of course, a metabolite of desoxycorticosterone and perhaps of other adrenal steroids but the amounts which have been recovered from probable adrenal precursors have been in the vicinity of 0.1 mg.

The small amount of pregnanediol recovered during the cycles shown in Figures 4 and 10 leads to speculation concerning a "poorly functioning" corpus luteum. The low yields obtained in these two cycles were not thought to be related to the specimens having been forced through a Gorbman filter, inasmuch as other specimens similarly treated showed no such consistent reduction in recovery. The concept of a corpus luteum deficient in progesterone production has been expressed by Brewer and Jones (21) who noted the presence of a histologically normal corpus luteum in the presence of an endometrium that was consistent with an anovulatory cycle. It should be pointed out, however, that in the present state of knowledge of progesterone metabolism it is presumptuous to assume that a low pregnanediol yield is indicative of deficient progesterone production. There is no definite evidence to show that pregnanediol recovery can be interpreted as a quantitative measurement of the amount of progesterone in the body.

SUMMARY

In normal menstrual cycles the excretion of urinary pregnanediol when determined in the free form shows no constant relationship to basal temperature curves. Pregnanediol was found in the urine, before, during, and after the temperature rise. The excretion of pregnanediol in normal subjects after the intramuscular injection of 60 milligrams of progesterone has been shown to be fairly constant, pregnanediol being found in the urine within twenty-four hours after administration. The lack of correlation between temperature change and pregnanediol excretion suggests that the

metabolic activity of progesterone as reflected in temperature rise occurs at variable rates in different individuals and in different cycles in the same subjects and reflects on the difficulty of detecting the time of ovulation by the use of temperature charts. Furthermore, the first appearance of pregnanediol in a normal cycle may not be assumed always to represent previous ovulation since the possibility of secretion of progesterone by preovulatory luteinization of the theca cannot be excluded.

Acknowledgment

The authors greatly appreciate the technical assistance of Miss Avery R. Grant.

REFERENCES

1. Venning, E. H., and Browne, J. S. L.: Studies on corpus luteum function. I. The urinary excretion of sodium pregnanediol glucuronidate in the human menstrual cycle, *Endocrinology* **21**: 711–721, 1937.
2. Stover, R. F., and Pratt, J. P.: Progestin studies: pregnanediol excretion, *Endocrinology* **24**: 29–34, 1939.
3. Wilson, R. B.; Randall, L. M., and Osterberg, A. E.: Studies on pregnanediol, *Am. J. Obst. & Gynec.* **37**: 59–74, 1939.
4. Hamblen, E. C.; Ashley, C., and Baptist, M.: Sodium pregnanediol glucuronide: The significance of its excretion in the urine, *Endocrinology* **24**: 1–12, 1939.
5. Cope, C. L.: The excretion of pregnanediol and the corpus luteum, *Clin. Sci.* **4**: 217–241, 1939–42.
6. Buxton, C. L.: Pregnanediol determination as an aid in clinical diagnosis, *Am. J. Obst. & Gynec.* **40**: 202–211, 1940.
7. Siegler, S. L., and Bauer, D.: An evaluation of the pregnanediol complex as an index of ovarian and uterine function, *Am. J. Obst. & Gynec.* **45**: 277–286, 1943.
8. Venning, E. H.: Gravimetric method for the determination of sodium pregnanediol glucuronidate (an excretion product of progesterone), *J. Biol. Chem.* **119**:473–486, 1937.
9. Venning, E. H.: Further studies on the estimation of small amounts of sodium pregnanediol glucuronidate in urine, *J. Biol. Chem.* **126**: 595–602, 1938.
10. Mack, H. C.; Parks, A. E., and McDonald, M.: Further observations on the pregnanediol precipitation test, *Harper Hosp. Bull.* **6**: 33–41, 1948.
11. Mack, H. C.; Parks, A. E., and McDonald, M.: The pregnanediol precipitation test. Further observations on clinical applications and technic, *J. Clin. Endocrinol.* **9**: 234–244, 1949.
12. Astwood, E. B.; Grady, A. B., and Wermer, O. S.: To be published.
13. Astwood, E. B., and Jones, G. E. S.: A simple method for the quantitative determination of pregnanediol in human urine, *J. Biol. Chem.* **137**: 397–407, 1941.
14. Marrian, G. F., and Gough, N.: Isolation of pregnane-3(α)-ol-20-one from the hydrolysis products of sodium pregnanediol 'glucuronidate,' *Nature, London* **157**: 438, 1946.
15. Gorbman, A.: Ultrafiltration of urine for collection and biological assay of excreted hypophyseal hormones, *Endocrinology* **37**: 177–190, 1945.
16. Tompkins, P.: Use of basal temperature graphs in determining date of ovulation, *J.A.M.A.* **124**: 698–700, 1944.

17. Davis, M. E.: The clinical use of oral basal temperatures, *J.A.M.A.* **130:** 929–932, 1946.
18. Greulich, W. W.: The reliability of "basal" body temperature changes as an index of ovulation in women, *Tr. Am. Soc. for Study of Sterility* pp. 76–97, 1946.
19. Farris, E. J.: Temperature compared with rat test for prediction of human ovulation, *J.A.M.A.* **138:** 560–563, 1948.
20. Davis, M. E., and Fugo, N. W.: The cause of physiologic basal temperature changes in women, *J. Clin. Endocrinol.* **8:** 550–563, 1948.
21. Brewer, J. I., and Jones, H. O.: Studies on the human corpus luteum: histologic variations in corpora lutea and in corpus luteum; endometrial relationship at the onset of normal menstruation, *Am. J. Obst. & Gynec.* **54:** 561–575, 1947.

AN IMPROVED PROCEDURE FOR THE QUANTITATIVE ESTIMATION OF URINARY PREGNANEDIOL

ROBERT E. HOYT, PH.D. AND MILTON G. LEVINE, PH.D.*

From the Institute of Experimental Medicine, College of Medical Evangelists, Los Angeles, California

THE estimation of urinary pregnanediol has been recommended as a test for pregnancy, and as an aid in predicting the fate of threatened abortion. In addition, it has been suggested that threatened abortion associated with diminished pregnanediol excretion may be averted by adequate progesterone therapy.

The widespread interest in this determination has resulted in the description of various procedures adapted to clinical use. Among them are the methods of Guterman (1), Sommerville, Gough, and Marrian (2), Rabinovitch (3), and Huber (4). These are derived to a greater or lesser extent from the procedure of Guterman, who has combined the methods of Astwood and Jones (5) and of Talbot *et al.* (6) in a test suited to routine use.

In our use of Guterman's method over a period of time, we have introduced certain modifications which seem sufficiently important to warrant their being reported. In addition, we have determined the percentage recovery under the conditions of our procedure, so that a quantitative determination of pregnanediol excretion can readily be made. Some observations dealing with the rate of excretion of pregnanediol were obtained during the progress of this work, and are included in this report.

I. MATERIALS AND METHOD

A. Materials

1. "Pure" toluene redistilled over C. P. potassium hydroxide pellets.
2. C. P. hydrochloric acid.
3. Reagent zinc metal granules (10 mesh).
4. C. P. sodium hydroxide.
5. C. P. potassium hydroxide pellets.
6. C. P. acetone.
7. C. P. sulfuric acid (97.1 per cent).
8. Optically matched pyrex culture tubes (18×150 mm.).

Received for publication June 1, 1949.

* We wish to acknowledge the technical assistance of John C. Sinclair, B. S. The clinical specimens were supplied by Dr. A. R. Abarbanel.

B. Method

1. Hydrolysis:

The volume of the urine specimen is measured, and a sample is removed for hydrolysis. It has been our practice to obtain the specimen over as long a period as possible and to hydrolyze one fifth of the volume. The sample is placed in a Florence flask of a suitable size together with 15 per cent of its volume of concentrated hydrochloric acid, 30 ml. toluene previously redistilled over potassium hydroxide pellets, and 3–6 zinc granules. The volume of the flask should be such that the toluene layer is near the middle of the flask. A single-hole cork stopper bearing an inverted 5 ml. volumetric pipette is fixed tightly in the neck of the flask, which is refluxed on a steam bath for thirty-five minutes. The bath should be kept out of any cool air streams; active bubbling from the granules should occur, and condensation on the walls of the flask should be noted. At the end of the period of hydrolysis, the flask is cooled in running water and decanted into a separatory funnel. The bottom (water) layer is drawn off and discarded.

2. Removal of acid impurities:

The toluene layer is washed twice by swirling vigorously with two 20 ml. portions of 1.0 N sodium hydroxide. (We have found that the 1.0 N solution separates more effectively and rapidly than does the 0.1 N sodium hydroxide used by Guterman. In addition, we have not found the additional washing with distilled water to be beneficial.) After the second wash, a five-minute period is allowed for separation.[1] A 25 ml. portion of the toluene layer is drawn off from above with a volumetric pipette and is placed in a 125 ml. Erlenmeyer flask. At this point the extract may be left overnight if desired.

3. Removal of impurities by potassium hydroxide treatment:

The flask containing the toluene is placed on a hot plate and brought to a gentle boil. If the proper heat is maintained, most of the toluene vapors will condense on the neck and walls of the flask, but it may be necessary to add small amounts of toluene occasionally. When the toluene has boiled for two or three minutes, a pellet of potassium hydroxide is added, followed by additional pellets at five- to ten-minute intervals until the toluene extract is clear and the last pellet remains white. The flask is stoppered and allowed to cool somewhat. The toluene is filtered through a fine fritted glass filter, the flask and sediment being rinsed with 5 ml. of toluene which is added to the extract by way of the filter. The toluene extract is then returned to the hot plate and evaporated to dryness, the final stages being carried out in a water bath under a stream of dry clean air. Overheating at this point must be avoided. A gray crystalline residue indicates the presence of pregnanediol.

4. Precipitation of pregnanediol:

The residue is dissolved in 2.5 ml. of acetone. The flask is placed on the hot plate and 10 ml. of 0.1 N sodium hydroxide is added. Heating is continued until vapors begin to condense on the sides of the vessel. The flask is removed from the hot plate, stoppered with a cork wrapped in waxed paper, and placed in the 37°C. incubator for two hours. A gray flocculent precipitate at this point indicates the presence of pregnanediol. We have found that pregnanediol in excess of 0.2 mg. gives an observable precipitate.

5. Colorimetric estimation of pregnanediol:

The contents of the flask are transferred to a 30 ml. medium porosity fritted glass filter, and gentle suction is applied. The flask and filter are washed with 10 ml. of a 5 per

cent solution of acetone in water. After the fluid has passed through the filter, strong suction is applied to dry the disc. The filter is inverted on a hot plate set at "low" for a few minutes and then fixed in a colorimeter tube with suction attached. (A small two-hole rubber stopper is suitable here.) Ten ml. of concentrated sulfuric acid is added to the funnel and allowed to stand two or three minutes while the color is developing. The acid is then filtered directly into the colorimeter tube, with occasional gentle swirling of the filter. The contents of the tube are mixed by tipping and read at 430 mμ against a blank of concentrated sulfuric acid. The color of the completed test should be a clear sparkling yellow-orange. The imperfect elimination of impurities may lead to the development of a dingy, degraded color which can be recognized by inspection, and which will give excessively high readings on the colorimeter.

6. Calculations:

As will be shown, the only significant loss of pregnanediol, after the stage of hydrolysis and extraction, occurs in step 4, where the precipitation of the steroid from the acetone-sodium hydroxide solution is incomplete. We have found this loss to be from 0.08 to 0.1 mg., so that this amount represents the limiting value of the method. This figure must also be added to the observed value in calculating the amount of pregnandiol. The formula is (reading of unknown/reading of standard × concentration of standard + 0.08) × 6/5 = mg. pregnanediol per sample of urine. This value must be multiplied by a dilution factor which is determined by the size of the sample removed for extraction.

II. QUANTITATIVE RECOVERY OF PREGNANEDIOL AT VARIOUS STAGES

A. Simultaneous hydrolysis and extraction of sodium pregnanediol glucuronide.

Crystalline sodium pregnanediol glucuronide[1] in solution was added to diluted pregnancy urine. The urine was hydrolyzed for thirty-five minutes over a steam bath in the presence of 15 per cent hydrochloric acid (by volume). After cooling, the toluene fraction was washed with 1.0 N sodium hydroxide and cleared by the addition of potassium hydroxide pellets. The clear toluene was filtered and evaporated to dryness. Ten ml. of sulfuric acid was added to the residue and the resulting colors were read at 410 mμ. Readings in density units and recoveries are shown in Table 1. From these values it is seen that a loss of 17 per cent occurs during hydrolysis and extraction. We have not found that increasing the time of hydrolysis up to one and one-half hours results in an increased recovery of pregnanediol. These values agree well with those reported by Astwood and Jones (5).

B. Recovery of pregnanediol by acetone-alkali precipitation.

Two-tenths mg. and 0.4 mg. samples of pregnanediol were dissolved in 2½ ml. of acetone and precipitated by the addition of 10 ml. of N/10

[1] We are indebted to Ciba Pharmaceutical Products, Inc., and to Ayerst, McKenna & Harrison, Ltd., for generous supplies of pregnanediol and sodium pregnanediol glucuronide.

TABLE 1. RECOVERY OF SODIUM PREGNANEDIOL AS PREGNANEDIOL, FOLLOWING COMBINED HYDROLYSIS AND EXTRACTION

Mg. of sodium pregnanediol glucuronide added	Reading in Coleman density units	Difference between blank and weighted sample	Sodium pregnanediol glucuronide equivalence	Per cent recovered
0	.35 .35 } .34 .33			
0.414	.66 .69 } .67	.33	.34	83.1
0.718	.92	.56	.60	83.3

sodium hydroxide. The colors developed were read in Coleman density units. The average loss is 0.13 density units, equivalent to 0.08 mg. pregnanediol. This loss appears to be constant, and is due to the solubility of pregnanediol in the acetone-alkali mixture. It may be decreased by reducing the amount of acetone used, but when this is done, false positive readings occasionally result, probably due to the decreased solubilities of other compounds present.

C. Effect of slow cooling of the precipitating mixture.

Our observations confirm those of Sommerville, Gough and Marrian (2) in regard to the rate of cooling of the acetone-alkali containing the precipitating pregnanediol. We have also compared 1.0 N with N/10 sodium hydroxide for use in this step, and have found that the weaker alkali and the precipitation at 37 C. with slow cooling give noticeably lower blanks with no decrease in pregnanediol recovery. In these experiments pregnanediol was added to cleared extracts of male urine.

D. Use of potassium hydroxide pellets in clearing the extract.

Nine Erlenmeyer flasks containing 0.2 mg. of pregnanediol in 25 ml. of toluene were prepared and arranged in three sets; one set received no

TABLE 2. RECOVERY OF PREGNANEDIOL BY ACETONE-ALCOHOL PRECIPITATION

Mg. of pregnanediol added	Recovery (Coleman density units)		
	Theoretic	Actual	Loss
0.2	0.30	0.195, 0.19	0.108
0.4	0.63	0.48, 0.48	0.15

TABLE 3. EFFECT OF POTASSIUM HYDROXIDE CLEARING ON RECOVERY OF 0.2 MG. OF PREGNANEDIOL

Potassium hydroxide pellets added	Pregnanediol recovered (Coleman density units)
None	.30
	.28
	.30
Two, in thirty minutes	.27
	.28
	.27
Four, in sixty minutes	.27
	.28
	.31

potassium hydroxide; the second, one pellet of potassium hydroxide every fifteen minutes for one-half hour; and the third, one pellet each fifteen minutes for one hour. Sets 2 and 3 were filtered; all three were dried and 10 ml. of sulfuric acid was added to each. The results, in density units, are shown in Table 3.

These values show that heating the pregnanediol in the presence of potassium hydroxide pellets does not result in any appreciable loss of activity.

III. COLLECTION OF SAMPLES OF URINE

A. Effects of storage of urine.

Since urine samples without preservative are frequently received in the laboratory, we have investigated the effect of storage of contaminated urine upon pregnanediol levels. A specimen of prenatal urine to which no preservative was added was sampled at intervals for a period of one week. The resulting pregnanediol levels are shown in Table 4.

TABLE 4. PREGNANEDIOL RECOVERY AFTER STORAGE OF URINE AT ROOM TEMPERATURE WITHOUT PRESERVATIVE

No. of days urine stored	Pregnanediol recovered (Coleman density units)	Per cent decrease
0	1.48	—
1	1.44	2.7
5	1.26	14.8
6	1.24	16.3
7	0.96	35.1

TABLE 5. RATE OF EXCRETION OF URINARY PREGNANEDIOL

Date	Patient	Time	No. of hours	Urine volume, cc.	Mg. of pregnanediol excreted
1-27-49	Morgan	9:30 A.M.– 4:30 P.M.	7	850	1.15
		4:30 P.M.– 8:00 A.M.	15½	890	1.43
2- 9-49	Morgan	6:30 P.M.– 7:15 A.M.	12¾	560	1.47
		7:15 A.M.– 6:00 P.M.	10¾	790	5.11
12- 5-48	Chiprin	8:35 P.M.– 3:25 A.M.	6½	820	12.8
		3:25 A.M.– 8:35 A.M.	6	410	6.25
1-20-49	Chiprin	8:30 P.M.–11:00 P.M.	2½	800	5.46
		11:00 P.M.– 7:00 A.M.	8	880	4.76
1-27-49	Chiprin	10:30 P.M.– 5:30 A.M.	7	830	8.56
		5:30 A.M.– 8:45 A.M.	3¼	390	5.66
2- 3-49	Chiprin	10:00 P.M.– 1:00 A.M.	3	570	13.64
		1:00 A.M.– 7:00 A.M.	6	450	10.45
12- 9-48	Little	6:15 P.M.– 7:30 P.M.	1¼	110	2.55
		7:30 P.M.–11:15 P.M.	3¾	250	5.46
		11:15 P.M.– 4:00 A.M.	4¾	335	5.01
12-10-48	Little	4:00 A.M.– 7.45 A.M.	3¾	195	3.85
		7:45 A.M.–11:00 A.M.	3¼	130	1.98
12-12-48	Little	7:35 P.M.–10:50 P.M.	3¾	270	10.05
		10:50 P.M.– 3:20 A.M.	4½	400	8.73
		3:20 A.M.– 7:50 A.M.	4½	165	10.94
		7:50 A.M.– 9:50 A.M.	2	100	4.28
		9:50 A.M.–12:20 P.M.	2½	115	4.92
		12:20 P.M.– 3:40 P.M.	3⅓	100	6.94
		3:40 P.M.– 6:40 P.M.	3	80	4.82
		6:40 P.M.– 8:00 P.M.	1⅓	75	4.26
8- 5-49	Rabinowitz	7:30 P.M.– 8:15 A.M.	12¾	280	3.88
		8:15 A.M.–10:00 A.M.	1¾	155	0.78
		10:00 A.M.– 2:00 P.M.	4	245	1.86
		2:00 P.M.– 7:30 P.M.	5½	200	1.46

The recovery of pregnanediol is seen to decrease with storage. Specimens to be used for quantitative recovery should be collected with a little toluene

in the flask as a preservative unless the hydrolysis is to be carried out within twenty-four hours after collection.

B. Rate of excretion at various times.

Patients frequently object to the difficulties involved in the collection and transportation of a 24-hour urine sample. This is particularly noticeable when studying pregnancies which occur in women subject to habitual abortion, in whom it is desirable to sample the urine at frequent intervals. In an effort to determine the value of samples collected over a shorter period of time, the rate of excretion at different periods during the day was determined for a number of pregnant women. Representative data are shown in Table 5.

It may be seen from these results, and especially from the 12-12-48 series of patient Little, that the excretion of pregnanediol does not occur at a uniform rate. The amount excreted during any period appears to be independent of the time of day or volume of urine. The 24-hour excretion of pregnanediol cannot be calculated from any single voided sample, but can be obtained only by sampling a 24-hour specimen.

SUMMARY

1. An improved procedure for the estimation of urinary pregnanediol is described, with analyses showing recoveries obtained at the different stages. The principal sources of loss are in the initial step of hydrolysis and extraction where a recovery of 80 to 85 per cent may be expected, and in the precipitating step where a loss of 0.08 mg. occurs. This is due to solubility of the pregnanediol in the acetone-alkali mixture.

2. No uniform excretion of pregnanediol was found to occur in relation to volume of urine or the time of day during which the urine was collected.

REFERENCES

1. a) Guterman, H. S.: A human pregnancy test based upon a color reaction of pregnandiol in the urine, *J. Clin. Endocrinol.* **4**: 262, 1944.
   b) Guterman, H. S.: Further observations on the value of the pregnandiol test for pregnancy, *J. Clin. Endocrinol.* **5**: 407, 1945.
2. Sommerville, I. F.: Gough, N., and Marrian, G. F.: Quantitative determination of small amounts of pregnanediol in human urine, *J. Endocrinol.* **5**: 247, 1948.
3. Rabinovitch, J.: Rapid method for estimation of pregnanediol, *Nature, London* **161**: 605, 1948.
4. Huber, D.: Determination of pregnanediol in urine for diagnostic purposes, *Biochem. J.* **41**: 609, 1947.
5. Astwood, E. B., and Jones, G. E. S.: Simple method for quantitative determination of pregnanediol in human urine, *J. Biol. Chem.* **137**: 397, 1941.
6. Talbot, N. B.; Berman, R. A.; MacLachlan, E. A., and Wolfe, J. K.: The colorimetric determination of neutral steroids (hormones) in a 24-hour sample of human urine (pregnanediol, total, alpha and beta alcoholic, and non-alcoholic 17-ketosteroids), *J. Clin. Endocrinol.* **1**: 668, 1941.

THE GONADS IN THE LAURENCE-MOON-BIEDL SYNDROME.

THREE CASE REPORTS WITH ONE PARTIAL AUTOPSY

C. FRANCKE, M.D.

From the Department of Internal Medicine, Municipal General Hospital, The Hague, Holland

THE Laurence-Moon-Biedl syndrome is diagnosed in patients presenting the majority or all of the following heredofamilial deviations: obesity, hypogonadism, retinitis pigmentosa, mental deficiency and polydactylism or syndactylism. The syndrome is not generally considered to be a definite disease entity. Warkany and co-workers (1, 2) demonstrated that this syndrome is only a more or less frequently occurring combination of certain heredofamilial deviations. Incomplete or transitional forms and supercomplete forms were found to be more numerous than typical ones. Apparently several features may be absent or replaced by others and in the literature opinions differ as to which are the cardinal symptoms required to justify the diagnosis of Laurence-Moon-Biedl syndrome.

Those sharing Warkany's opinion will not have been surprised by Anderson's statement (3) concerning the 6 cases of this syndrome, autopsy reports of which have been published: "unfortunately the lack of uniformity in the morbid changes of these cases has prevented diagnostic correlation." No constant diencephalic or pituitary lesions had been found. According to Anderson's review "the findings in the hypothalamus have not been remarkable." In 2 cases (including Anderson's patient) there was a predominance of basophilic cells in the anterior pituitary.

Hypogonadism is the endocrine disorder most frequently described in the syndrome, probably because most clinicians require the presence of this lesion before labelling a case as Laurence-Moon-Biedl syndrome.

In textbooks, *e.g.*, Selye's (4), the syndrome is generally placed under the heading "Anterior Lobe Hypofunction." The hypogonadism is presumed to be hypogonadotropic, caused by selective failure of pituitary gonadotropin production.

Roth (5) has been the first to demonstrate the presence of hypogonadotropic hypogonadism by urinary hormonal assays as well as by testicular biopsies. His 4 patients lacked retinitis pigmentosa, mental retardation, polydactylism or syndactylism and only 1 of them was obese, which is an additional illustration of the rather vague definition of the Laurence-Moon-Biedl syndrome.

Received for publication June 29, 1949.

The description of the gonads in the 3 cases in which autopsy data have been available (*viz.*, Anderson (3)) point in the same direction.

However, in many cases labelled as Laurence-Moon-Biedl syndrome and presenting all other characteristics of the pentad, hypogonadism has been absent. Warkany, Frauenberger and Mitchell listed many of them in their review (1). A patient of Biemond's series (6) (she even gave birth to a normal child) and the fifth of Roth's patients (an adult Negro whose testicular biopsy specimen was entirely normal) also belong to this category, as well as the following 2 patients in whom urinary hormonal assays (F.S.H., 17-ketosteroids and corticosteroids) have been performed in this Department. The patients were sisters; their parents, first cousins. Both patients presented all features of the Laurence-Moon-Biedl syndrome but the hypogonadism, if any, was very slight and the urinary F.S.H. excretion normal.

J. M., a 51-year-old single woman, had always been very obese. At 16 years of age her weight was 96 Kg. Menarche occurred at 16, and the menopause at 45 years. Menstruation was reported as having been normal. She was hospitalized in the fall of 1947 because of progressive weight loss, polyuria and glycosuria. On admission she was in diabetic precoma. Vomiting, marked dehydration and acidosis were present. Other findings included: mental deficiency, microcephaly, squint, retinitis pigmentosa, polydactylism (hands and the right foot) and abdominal striae. There was a marked dorsal kyphosis; body length was 1.53 M. and span 1.58 M. Genitals: The vaginal opening was narrow and there was an intact, resistant hymen. The uterus could not be palpated on rectal examination. The breasts and the type and distribution of scalp, pubic and axillary hair were normal.

During the first months it proved difficult to keep the urine free from acetone. Afterwards the diabetes was under control with 22 U. of P.Z.I. One year after discharge the nutritional state was satisfactory (weight 60.9 Kg). At that time urinary hormonal assays were made. F.S.H. excretion in twenty-four hours amounted to 200 M.U. (Varney, Kenyon and Koch's method (7)), 17-ketosteroid excretion was 5.9 mg. and corticoid excretion (Corcoran and Page's method (8)), 1.68 mg. in twenty-four hours.

T. M., a 45-year-old single woman, had always been very obese. When first seen, her height was 1.52 M. and weight 87 Kg. Recently reducing measures had been instituted elsewhere, apparently consisting of thyroid medication. She was glad to have lost 5 Kg. in the preceding four months but complained about palpitation, substernal pain on effort and extensive edema of both legs. Mental deficiency was evident. Retinitis pigmentosa, squint and polydactylism of the left foot were present. Breasts and distribution of scalp, pubic and axillary hair were normal. Menstruation was regular and normal. Blood sugar values were normal. The F.S.H. excretion in twenty-four hours amounted to 30 M.U., 17-ketosteroid excretion was 8.3 mg., and corticosteroid excretion, 0.47 mg. in twenty-four hours.

Of course, the amount of formaldehydogenic corticosteroids excreted in the urine of patient J. M. (upper limit of normal) cannot be looked upon as evidence of a steroid genesis (9) of her diabetes. Moreover, she had a strong tendency to acidosis. The diabetes was kept under control with 22

units of protamine zinc insulin. An aunt died of diabetes mellitus and the patient had hypertension (170/100).

In patients presenting the Laurence-Moon-Biedl syndrome, hypogonadism and eugonadism have been found. It would not be surprising if hypergonadotropic hypogonadism was present in some of them. Hypergonadotropic hypogonadism may occur as a heredofamilial deviation. According to Reifenstein (cited by Heller and Nelson (10)) this is probably so in cases of puberal seminiferous tubule failure.

Recently in this Department a patient suffering from the Laurence-Moon-Biedl syndrome was observed whose autopsy findings were consistent with a diagnosis of puberal seminiferous tubule failure.

P. S., a 45-year-old man, was first admitted to this hospital in 1937. At that time he was suffering from severe attacks of bronchial asthma. Obesity, eunuchoid proportions, a high pitched voice, hypogonadism, unilateral cryptorchidism, retinitis pigmentosa and mental retardation were noted. Polydactylism was absent. An elder brother and a sister (both deceased) were reported to have shown similar abnormalities. Other findings included a normal glucose tolerance curve and a B.M.R. of +10 per cent. A diagnosis of incomplete Laurence-Moon-Biedl syndrome was made. After discharge, the patient failed to return to the Out-patient Department. In November 1948 he was readmitted in uremic coma, reported to be of several days' duration. The patient was still very obese; the fat was mainly deposited in the shoulders, chest, abdomen and thighs. There was gynecomastia (fat). Striae were present on the abdomen and on the shoulders. Scalp hair was sparse, and the recession less than normal. Axillary hair was sparse and facial hair was almost absent. Pubic hair was sparse and showed a female type of distribution. There was no other body hair. The skull was scaphoid and microcephalic. There was extensive vitiligo of the hands, face, feet and scrotum. The penis was short (2 cm.) and thin. The right half of the scrotum was underdeveloped and the right testis was palpable in the inguinal canal. The left testis was about three quarters of normal size and rather firm. The prostate gland was small and the seminal vesicles were not felt on rectal examination.

Anuria was present and peritoneal dialysis was considered. However, before this had been instituted the patient died.

Partial autopsy was obtained. Hypertrophy of the right and left ventricles, fibrinous pericarditis and arteriolar nephrosclerosis were found (final stage, the weight of both kidneys taken together being 74 Gm.). Other findings were a normal pancreas and adrenals and a small prostate gland and seminal vesicles. In the left testis, hyalinization and thickening of the basement membrane and of the tunica propria of the seminiferous tubules was seen. All stages, marked to slight, were encountered. In all tubules spermatogenesis was impaired: spermatozoa or sperm head stages were absent. Several tubules contained only Sertoli cells or Sertoli cells and spermatogonia. A few tubules were completely sclerosed and hyalinized. The number of Leydig cells appeared to be increased and there was clumping of Leydig cells, which seemed in poor condition, small, and containing large quantities of pigment granules. The interstitial tissue contained an increased number of fibrocytes and fat cells and many eosinophilic leukocytes were present. The right testis was almond sized. The degree of sclerosis and hyalinization of the seminiferous tubules was even more pronounced here than in the left testis and the aggregations of Leydig cells were more prominent. There was no permission to examine the head.

In this case, owing to anuria, urinary hormonal assays could not be performed. Consequently, the existence of an increased F.S.H. excretion

could not be demonstrated. However, the history as well as the anatomic findings are consistent with a diagnosis of late puberal seminiferous tubule failure with failing Leydig cell function.

Apparently, patients suffering from the Laurence-Moon-Biedl syndrome may present:

1. Hypogonadotropic hypogonadism, as a congenital familial abnormality.
2. Eugonadism.
3. Hypergonadotropic hypogonadism, probably also as a congenital familial deviation.

Accordingly, hypogonadism in the Laurence-Moon-Biedl syndrome is not necessarily a result of pituitary hypofunction. This tends to confirm Warkany's view that the Laurence-Moon-Biedl syndrome is not a disease entity but a more or less frequently occurring combination of heredofamilial deviations. The findings in our third case suggest that one of these heredofamilial deviations may consist of a primary testicular abnormality without evidence of pituitary hypofunction.

SUMMARY

Patients suffering from the Laurence-Moon-Biedl syndrome may present hypogonadotropic hypogonadism (as a congenital familial abnormality), eugonadism, or hypergonadotropic hypogonadism (probably also as a congenital familial deviation). Two case histories (with urinary hormonal assays) of the eugonadal group are reported. One of these patients was a diabetic. In a third patient the autopsy findings were strongly suggestive of hypergonadotropic hypogonadism.

REFERENCES

1. Warkany, J.; Frauenberger, C. S., and Mitchell, A. G.: Heredofamilial deviations. I. The Laurence-Moon-Biedl syndrome, *Am. J. Dis. Child.* **53**: 455–470 (Feb.) 1937.
2. Warkany, J., and Weaver, T. S.: Heredofamilial deviations. II. Enlarged parietal foramens combined with obesity, hypogenitalism, microphthalmos and mental retardation, *Am. J. Dis. Child.* **60**: 1147–1154 (May) 1940.
3. Anderson, N. L.: The Laurence-Moon-Biedl syndrome: case report with complete autopsy, *J. Clin. Endocrinol.* **1**: 905–911 (Nov.) 1941.
4. Selye, H.: Textbook of Endocrinology. Montreal, Acta Endocrinologia, 1947, p. 272.
5. Roth, A. A.: Familial eunuchoidism: the Laurence-Moon-Biedl syndrome, *J. Urol.* **57**: 427–445 (March) 1947.
6. Biemond, A.: Het syndroom van Laurence-Biedl en een aanverwant nieuw syndroom, *Nederl. tijdschr. v. geneesk.* **78**: 1801–1809 (Apr.) 1934.
7. Varney, R. F.; Kenyon, A. T., and Koch, F. C.: An association of short stature, retarded sexual development and high urinary gonadotropin titers in women, *J. Clin. Endocrinol.* **2**: 137–145 (March) 1942.

8. Corcoran, A. C., and Page, I. H.: Methods for the chemical determination of corticosteroids in urine and plasma, *J. Lab. & Clin. Med.* **33**: 1326–1333 (Oct.) 1948.
9 Sprague, R. G.; Hayles, A. B.; Mason, H. L.; Power, M. H., and Bennett, W. A.: "Steroid diabetes" associated with Cushing's syndrome and excretion of 17-hydroxycorticosterone (compound F) in urine; metabolic studies, *J. Lab. & Clin. Med.* **33**: 1472–1473 (Nov.) 1948.
10. Heller, C. G., and Nelson, W. O.: Classification of male hypogonadism and a discussion of the pathologic physiology, diagnosis and treatment, *J. Clin. Endocrinol.* **8**: 345–366 (May) 1948.

BOOK REVIEW

Hormones and Behavior: A Survey of Interrelationships between Endocrine Secretions and Patterns of Overt Response. By Frank A. Beach, with a foreword by Earl T. Engle. XIV + 368 pages. Price, $6.50. New York, Paul B. Hoeber, Inc., 1948.

The relationships between behavior—especially reproductive behavior—and the hormones is a matter of world-wide interest. Nevertheless, in no other field of biology is there evident more misapprehension and lack of clear appreciation of scientific fundamentals. As a matter of editorial knowledge, opinions of mostly well-informed writers range the entire gamut from an assumption that sexuality is essentially a matter of gonadal hormones to the opposite extreme position that, in man at least, hormones have little or nothing to do with the matter. There is corresponding need for such a book as that of Beach.

The author has been for years an astute investigator in this field, first as Chairman of the Department of Animal Behavior at the American Museum of Natural History and more recently as Professor of Psychology at Yale. He has conducted and published many researches on a variety of forms—fishes, amphibians, reptiles, birds, and mammals. With this comprehensive background he has mastered a large body of scattered literature including that on human and higher primate behavior.

In this monograph he deals with the hormonal influences on behavior in the larger sense but emphasizes especially reproductive behavior, including not only mating, but also nesting, nurturing, migration, and individual aggressiveness. The major topics that are dealt with include: courtship and mating; reversed mating behavior ("homosexuality"); oviposition, parturition, and parental behavior; migration; aggression and dominance; emotion; conditioning; locomotor activity; homeostasis; morphologic structuring as related to behavior; the role of nervous stimulation; developmental aspects; major sources of variability; and interpretation of hormonal effects. Although some of the material that is cited is of interest chiefly to the comparative psychologist, much of it pertains directly to the problems of the clinical endocrinologist. Indeed, the interests of the latter are kept in the foreground throughout the book.

Those who think of "sex" as merely a matter of simple cause-and-effect relationships to the formed gonadal hormones may profit especially from Beach's exposition of some of the complexities of mating behavior. The maturing organism must attain five major goals in order to achieve efficient reproduction. The anterior pituitary must begin to produce and release enough gonadotropic hormones; the gonads must have become adequately sensitive to these hormones; the accessory sex structures must respond in normal

adult fashion to the gonadal hormones; the secondary sex characters that are under hormone control must be reactive; and the behavior mechanisms responsible for courtship and mating must have become fully organized and responsive to the appropriate hormones. These factors may reach maturity at different rates and in different degrees of completeness. And even though the structural and physiologic mechanisms may all exist in normal status, the mechanisms may be rendered inoperative by psychologic factors.

It is argued persuasively that the reproductive mechanisms are determined by genetic factors and that the hormones are in actuality to be regarded not as primary stimuli to behavior nor as organizers of overt responses, but merely as facilitating agents which increase the reactivity of the specific neuromuscular systems to stimulation. In this regard "male" hormones and "female" hormones are in considerable degree interchangeable.

It is emphasized that libido, potency, and fertility are three independent functions, any one of which may exist in the absence of the other two, or any two in the absence of the other one. Several illustrations of this generalization are cited.

The interplay of hormonal, psychologic, and social factors in reproductivity is discussed in several passages. Contrary to common belief, social factors may play a determining role not only in man, but in lower forms as well.

In final emphasis it is pointed out that no behavior mechanism thus far investigated has been found to depend upon a single hormone. Endocrine control of a complete behavior pattern appears to be mediated by several intervening mechanisms rather than a single critical one. Different patterns depend in different degrees upon hormones. Any behavior response constitutes a reaction to internal and external stimuli: the characteristics are determined jointly by genetic constitution, previous excitation, and the chemical nature of the internal medium. Hormones may influence behavior through their effects 1) upon the total organism; 2) upon specific structures employed in particular response patterns; 3) upon peripheral receptor mechanisms; and 4) upon the integrative functions of the central nervous system.

When the work goes into later editions, as no doubt it will, it may be hoped that Beach will go a bit further into the philosophic aspects of the subject. His remarks upon the ineptitude of "tubular vision" in so involved a situation might well be supplemented by a consideration of the inutility of cause-and-effect logic itself, in dealing with phenomenology that actually represents complex equilibria, in which cause and effect are intermingled and often interchangeable. We need a new conceptualization in which what might be called biologic relativity is more explicitly regarded.

An omission for which the author can fairly be taxed is a consideration of the implications of the anterior pituitary–"target organ" relationship. The sex hormones in their feed-back controlling influences on the hypophysis play havoc with the simple quantitative cause-and-effect concepts that are implicit in much current discussion. The gonadal hormones are in considerable measure self-antidoting agents and the operation of this complication might well have been discussed.

The book is written in readable fashion. It is well indexed and is printed in attractive form. It closes with a working bibliography of 65 pages and, in the interest of the general reader, an eight-page glossary.

In more than the usual degree of literalness, this volume deserves a place in the library of every one who is technically interested in reproduction from any point of view. It is genuinely unique.

Postgraduate Assembly in Endocrinology Including Diabetes

Sponsored by

THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

and

THE AMERICAN DIABETES ASSOCIATION

MIAMI BEACH, FLORIDA RONEY PLAZA HOTEL APRIL 3–8, 1950

The faculty will consist of prominent researchers and clinicians in the field of endocrinology and metabolic disorders, from the United States and Canada.

The course will be a practical one of interest and value to the specialist and those in general practice. The program will consist of lectures, clinics and demonstrations. Ample time will be given to questions and answers at the end of each session, and registrants are encouraged to contact members of the faculty for individual discussions.

The Roney Plaza, one of Miami Beach's most delightful hotels, offers special convention rates to members of this assembly. This is an unusual opportunity for you and your family to enjoy a pleasant vacation and for you to participate in a highly instructive program of the latest advances in endocrinology and metabolism.

A fee of $75 will be charged for the entire course and the attendance will be limited to 100. REGISTRATION WILL BE IN THE ORDER OF CHECKS RECEIVED AND WILL CLOSE ON MARCH 3, 1950. Should there be an insufficient number of applicants to fill the course, the registration fee will be refunded immediately in its full amount.

Application for approval of this course has been made to the Veterans Administration. Veterans should make formal application to their local agencies on the appropriate form (1905e or 1950) as furnished by the V.A.

Please forward application on your letterhead, together with check payable to The Association for the Study of Internal Secretions, to Henry H. Turner, M.D., Secretary-Treasurer, 1200 North Walker Street, Oklahoma City 3, Oklahoma, before March 3, 1950. Further information and program will be furnished upon request.

Hotel reservations should be made directly with the Roney Plaza Hotel, Miami Beach, Florida, and the hotel advised that you are attending this Postgraduate Assembly.

The 1950 Meeting of the Association for the Study of Internal Secretions

The Thirty-Second Annual Meeting of The Association for the Study of Internal Secretions will be held at the Sir Francis Drake Hotel, Friday and Saturday, June 23 and 24, 1950, in San Francisco, California.

The Committee on Local Arrangements is comprised of Dr. Hans Lisser, Chairman, and Drs. Leslie L. Bennett, Roberto F. Escamilla, Minnie B. Goldberg, Gilbert S. Gordan, and Laurance W. Kinsell.

Hotel accommodations will be difficult to secure on short notice; therefore, members are urged to make their reservations at once. All requests must be addressed to: Dr. William Howard Rustad, American Medical Association (Hotel Committee), Room 200, Civic Auditorium, San Francisco, California.

The scientific sessions will be held in the Empire Room of the Sir Francis Drake, and registration will be on the same floor. The annual dinner will be held in the Empire Room on Friday, June 23, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Edward A. Doisy, M.D., St. Louis University School of Medicine, 1402 South Grand Avenue, St. Louis, Missouri, not later than *March 1, 1950. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program and journals of the Association.* Names of non-members who are co-authors must be followed by the words "by invitation," and the principal degree of each author.

Nominations for the Ayerst, McKenna and Harrison Fellowship, the Schering Fellowship in Endocrinology, the Squibb Award, and the Ciba Award should be made on special application forms which may be obtained from the Secretary-Treasurer, Henry H. Turner, M.D., 1200 North Walker Street, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than *March 15, 1950.*

The 1950 Awards of the Association for the Study of Internal Secretions

FELLOWSHIPS

The Ayerst, McKenna and Harrison Fellowship

The Ayerst, McKenna and Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin and in 1948 to Dr. Ernest M. Brown, Jr. Dr. Brown was re-elected for the Fellowship in 1949.

The Schering Fellowship in Endocrinology

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Lawrence Wilson.

Association Fellowships are designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $2,500, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

AWARDS

The E. R. Squibb and Sons Award

The E. R. Squibb & Sons Award of $1,000 was established in 1939, and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945

to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; and in 1949 to Dr. Herbert M. Evans. In 1943 no award was given. A special committee of five members of the Association selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

The Ciba Award

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; and in 1949 to Dr. George Sayers. The Award is for $1,200. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $1,800.

* * * * *

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on *special application forms* which may be obtained from the *Secretary*, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than *March 15, 1950.*

THE AMERICAN SOCIETY FOR THE STUDY OF STERILITY

ANNUAL MEETING

The next Annual Meeting of the American Society for the Study of Sterility will be held in the Sir Francis Drake Hotel, San Francisco, California, June 24, and 25, 1950.

AWARD

The American Society for the Study of Sterility is offering an Annual Award of $1,000 known as the Ortho Award for an essay on the result of some clinical or laboratory research pertinent to the field of sterility. Competition is open to those who are in clinical practice as well as to individuals whose work is restricted to research in basic fields or full time teaching positions. The Prize Essay will appear on the program of the 1950 meeting of the Society.

Full particulars may be obtained from the Secretary, Dr. Walter W. Williams, 20 Magnolia Terrace, Springfield 8, Massachusetts. Essays must be in his hands by *April 1, 1950.*

The 1950 Meeting of the American Goiter Association

The 1950 annual meeting of the American Goiter Association will be held in Houston, Texas, on March 9, 10, and 11. The famous Shamrock Hotel will be the headquarters and undoubtedly every effort will be made by the hotel officials to make the meeting a success and the visit to Houston a very pleasant one. It would be well to see that reservations are made at this time.

The President, Dr. Samuel Haines, and the Chairman of the Program Committee, Dr. Rulon Rawson, have planned an excellent program and a fine meeting is anticipated. It is hoped that there will be a large attendance of members.

The following is a preliminary program. The final program will be printed and mailed prior to the time of the meeting.

GEORGE C. SHIVERS, M.D.
Colorado Springs, Colorado,
Corresponding Secretary.

PRELIMINARY PROGRAM

Thursday—March 9

8:00 A.M.—*Registration*

9:00 A.M.—*General Session*

Diagnostic Methods in the Study of Human Thyroid Disease.

Dr. Elmer C. Bartels—Basal Metabolic Rate and Blood Cholesterol.
Dr. A. Querido—TSH in Differential Diagnosis of Hypometabolic States.
Dr. S. B. Barker—Blood Iodine.
Dr. Paul Starr—Blood Iodine.
Dr. Sidney Werner—I^{131} Tracer.
Dr. F. Raymond Keating, Jr.—I^{131} Tracer.
Discussion.
Guest Lecture.
Van Meter Award Paper.

2:00 P.M.—*General Session*

Exophthalmos.

Dr. Brown M. Dobyns—Pathologic Physiology and Etiology.
Dr. A. D. Ruedemann—Natural Life History of Exophthalmos.

Dr. E. Perry McCullagh—Medical Management of Exophthalmos.
Dr. James L. Poppen—Surgical Treatment of Exophthalmos.
Discussion.

Symposium on the Role of the Adrenal in Thyroid Disease.

Dr. W. L. Money or Dr. Reis.
Drs. Statland and J. Lerman.
Dr. George W. Thorn.

Friday—March 10

8:00 A.M.—*Registration.*

9:00 A.M.—*General Session.*

Symposium on the Treatment of Graves' Disease with I^{131}.

Drs. Clark, Gordon, Chapman and Blumgart.
Discussion.

The Toxic Effects of I^{131}.

Dr. Gorbman—Pathologic and Metabolic Consequences of High Radiation Dosages in Mice.
Brookhaven and Memorial Hospitals—Distribution of I^{131} in Human Beings.
Drs. Koch and Talbot—Porphyrins.

2:00 P.M.—*General Session*

Presidential Address.

Dr. Samuel F. Haines—The Influence of Research on the Modern Treatment of Exophthalmic Goiter.

Cancer of the Thyroid.

Dr. B. J. Duffy—Cancer of the Thyroid in Children.
Dr. Cattell or Dr. Frantz—Treatment of Cancer of the Thyroid.

4:00 P.M.—*Annual Business Meeting.*

Saturday—March 11

9:00 A.M.

Nontoxic (Sporadic or Endemic) Goiters.

Dr. I. Greenwald—Endemic Goiter: Deficiency, Intoxication or Infection.
Dr. George Curtis—Iodine Metabolism in Nontoxic Goiters.
Dr. F. Andrews—Title not announced.
Dr. J. Stanbury—Title not announced.
Dr. W. C. Thornell—Title not announced.